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CONTENTS

A MICROBIOLOGICAL ASSAY METHOD FOR THIAMINE

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It is generally recognized that the most useful and convenient means of quantitatively determining members of the vitamin B complex involve microbiological methods. Several methods of this type have been proposed for the assay of thiamine but each one in use today possesses certain inherent disadvantages that limit its value.

The fermentation method of Schultz, Atkin, and Frey (1, 2), based on the increased rate of alcoholic fermentation by yeast upon addition of thiamine increments, is not adaptable to large scale work. The number of assays that can be carried out is limited by the apparatus. In addition, the "pyrimidine" cleavage product is active, thus requiring two determinations for each product in order to obtain a true picture of the thiamine content of certain food materials. The method in which *Phycomyces blakesleeanus* is used as the test organism (3, 4) is time-consuming and is subject to error, since the organism is able to utilize the two moieties of the thiamine molecule. Both the "pyrimidine" and "thiazole" portions are active in the yeast growth method as proposed by Williams, McMahan, and Eakin (5); in addition, this organism is not sensitive to cocarboxylase. The microbiological method with a strain of *Staphylococcus aureus* as the test organism, as reported by West and Wilson (6), is also subject to error, since this organism is able to synthesize thiamine from the two cleavage products of the vitamin (7).

The purpose of this communication is to introduce a thiamine assay procedure which takes advantage of an organism for which neither cleavage product, alone or in combination, is active. As little as 0.01 millimicrogram of thiamine per ml. of medium can be quantitatively detected. Under the conditions tested, cocarboxylase is approximately 40 per cent more active than thiamine, calculated on a molecular basis, thus requiring an enzymatic hydrolysis for precise determinations in some food products.

EXPERIMENTAL

Organism—The test organism used is a strain of *Streptococcus salivarius* (Strain S20B). Members of this species are characterized by their ability to synthesize large amounts of a polysaccharide when grown on sucrose or raffinose agar (8, 9). These organisms can be serially cultured in a medium containing only chemically defined constituents, consisting of five B vita-

1

mins and seven amino acids (10). All strains tested required the addition of thiamine to the medium before growth would occur.

Stock cultures of the test organism are maintained in the form of stab cultures in a meat infusion agar plus 1 per cent tryptone, 0.1 per cent glucose, 0.2 per cent K_2HPO_4, 1.5 per cent agar, and excess $CaCO_3$. After growth the stab cultures are kept in the refrigerator. Stock cultures should be transferred every 6 weeks. Inoculums for assays are prepared by transferring the culture from the agar stab directly into the basal medium to which 10 millimicrograms of thiamine per 10 ml. have been added. The culture is incubated at 37° for 24 hours, or until good growth occurs, before being used to inoculate the assay tubes. An inoculum for the following day may be prepared by subculturing again in the same medium. Several

TABLE I

Basal Medium

Casein hydrolysate..	0.5 gm.
Thiamine-free yeast extract....................................	0.3 "
Glucose...	1.0 "
Phosphate buffer (0.4 M, pH 7.4).............................	10.0 ml.
Salt solution*..	1.0 "
Sodium thioglycolate..........	10.0 mg.
Uracil.............	0.5 "
Nicotinic acid..................	50.0 γ
Riboflavin...	50.0 "
Calcium pantothenate...........	50.0 "
Biotin (methyl ester).....	0.1 "
Distilled water to................................	100.0 ml.

* The stock solution of salts consists of 10 gm. of $NaCl$, 0.8 gm. of $MgSO_4$, 40 mg. of $FeSO_4 \cdot 7H_2O$, and 12 mg. of $MnCl_2$ in 100 ml. of distilled water.

tubes of medium to be used as inoculums may be made up at one time, but care should be taken to steam the medium for several minutes before use in order to insure prompt growth.

Basal Medium—The thiamine-free basal medium contains the ingredients listed in Table I. When sufficient thiamine is added to this medium, *Streptococcus salivarius* grows in intensity and rapidity equal to that in any ordinary laboratory medium.

Hydrolyzed Casein—A 10 per cent acid-hydrolyzed, vitamin-free casein solution is prepared in the usual manner with the use of H_2SO_4 and subsequent removal of the acid with $Ba(OH)_2$. Traces of thiamine, if present, are removed with norit (20 gm. per 100 gm. of casein) at pH 3.0. Since the test organism is able to synthesize tryptophane, it is unnecessary to replenish the hydrolyzed casein with this amino acid.

Thiamine-Free Yeast Extract—A 3 per cent thiamine-free yeast extract is prepared by the method of Williams, McMahan, and Eakin (5), with a few modifications. 6 gm. of Difco Bacto-yeast extract are dissolved in 200 ml. of water and autoclaved for 15 minutes. The pH is adjusted to 3.0, 10 gm. of fullers' earth are added, and the mixture is shaken for 30 minutes. The fullers' earth is then filtered out, and the filtrate adjusted to pH 1.0 and autoclaved another 15 minutes. After cooling, a second 10 gm. portion of fullers' earth is added and the mixture is shaken in a mechanical shaker overnight. The fullers' earth is again filtered out; 1.5 gm. of K_2HPO_4 are added to the filtrate, which is then adjusted to pH 7.4 and autoclaved again for 15 minutes. The resulting precipitate is filtered out, and the volume adjusted to 200 ml. and sterilized in convenient sized lots. The added phosphate serves the twofold purpose of destroying the remaining traces of thiamine and removing substances which would precipitate out in the complete medium.

The test organism will grow satisfactorily in the medium without the thiamine-free yeast extract but when this substance is omitted in the assay procedure there is occasionally an erratic response to increasing quantities of added thiamine and food substances, usually resulting in high assay values. Yeast and tissue extracts freed of thiamine by other means (adsorption with norit; sulfite treatment (2)) have proved to be inferior supplements.

Other Medium Constituents—A stock solution of phosphate buffer is made up by mixing equal volumes of 0.4 M K_2HPO_4 and KH_2PO_4 and adjusting to pH 7.4. 10 ml. of this buffer solution are used to prepare 100 ml. of medium.

Stock solutions of the salt mixture, reducing agent, and vitamins are conveniently made up separately in such concentrations that 1 ml. of each is sufficient per 100 ml. of medium. All stock solutions are sterilized and stored in the refrigerator.

Procedure

Assays are carried out in standard 6 inch bacteriological test-tubes which have been selected to be free from scratches and are of constant diameter (16 mm.). These tubes are conveniently supported in a metal rack that may be autoclaved.

The simplest procedure is to make up sufficient medium in concentrations double that necessary in the final preparation and to adjust the pH to approximately 7.4 before 5 ml. amounts are placed in each tube. Distilled water is added from a specially cleaned burette in such quantities that each tube will contain a final volume of 10 ml. after the test substance is added.

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Since thiamine is partially destroyed on heating in alkaline, and even in

neutral solutions, it is necessary to add the standard vitamin solution, as well as the test substance, aseptically to the medium after autoclaving. A standard thiamine solution is prepared by weighing 10 mg. of thiamine, which has been desiccated to constant weight, into 100 ml. of 0.1 M acetate buffer, pH 4.5. This stock solution may be sterilized by autoclaving 15 minutes and stored in the ice box. The standard solution should be changed at occasional intervals.

In order to establish a curve for assay an aliquot of the stock thiamine solution is diluted aseptically in sterile distilled water to a concentration of 1 millimicrogram per ml. Increasing quantities of this diluted solution are added to the sterilized tubes of medium in duplicate so that tubes containing 0, 0.1, 0.2, 0.4, 0.7, 1.0, and 2.0 millimicrograms per tube (10 ml.) are obtained. Samples for assay are added in other tubes in volumes estimated to contain between 0.1 and 0.6 millimicrogram of thiamine. Volumes up to 5 ml. may be used.

0.5 ml. of the inoculum prepared as described previously is mixed with 10 ml. of sterile saline solution. 1 drop of this diluted mixture is used to inoculate each tube of medium. The tubes are then rotated in order to obtain a uniform mixture of thiamine, or test substance, and inoculum throughout the medium. The tubes are incubated at 37° for 24 hours, after which the growth response is measured. Longer periods of incubation do not appreciably alter the assay values.

The sensitivity range may be broadened considerably if the glucose is added aseptically along with the thiamine, or test substance, after the basal medium has been autoclaved. This procedure affords a much greater maximum growth, since the initial pH of the medium is maintained at approximately 7.4 during autoclaving. By following this procedure browning of the medium, which might be of hindrance if growth response is to be measured by titrating the developed acid, is avoided.

Acetate buffer, either alone or in combination with phosphate, did not prove to be superior to phosphate as a buffer.

The need for scrupulously cleaned glassware and materials cannot be overstressed because of the extreme sensitivity of the test organism toward thiamine. It is advisable to use plastic or aluminum caps that can be cleaned well between runs. If cotton plugs are used, they should be covered with a square of cheese-cloth before being inserted into the tubes.

The medium should be made up on the day it is used. A standard curve should be established for each day's run.

Measurement of Growth Response—Turbidimetric measurements with a photoelectric colorimeter have been found to be more satisfactory for measuring growth response than titrating the developed acid, since the maximum quantity of acid produced is not so large as that which can be attained with

Lactobacilli. Good results have been obtained, however, by titrating with 0.05 N NaOH, with brom-thymol blue as an indicator.

Results

A typical curve resulting from response to thiamine is shown in Fig. 1. The thiamine content of the test substance may be determined by compar-
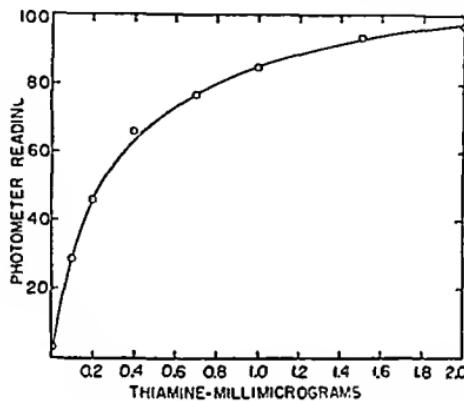
FIG. 1. Growth response of *Streptococcus salivarius* to thiamine

TABLE II

Comparative Thiamine Assays of Various Materials

Material	*Streptococcus salivarius*	*Phycomyces blakesleeanus*
	γ per gm. or ml.	γ per gm.
Dried milk, A	4.3	4.5
" " B	4.3	4.7
Whole wheat flour	6.6	4.2
Dried turnip tops	4.5	4.4
" tomatoes	11.1	8.2
Raw skim milk, A	0.51	
" " " B	0.55	
" whole milk	0.55	
Pasteurized whole milk	0.54	

ing the growth response to that produced by thiamine on the standard curve. The average of values obtained at two or more levels is used. Values taken at the extreme lower or upper flat portion of the curve may result in considerable error.

A few results from a number of tests on various food products are shown in Table II. The milk samples were prepared by placing 1 ml. of the milk in 100 ml. of 0.1 M acetate buffer, pH 4.5, and autoclaving for 15 minutes.

The sterile suspension was then diluted aseptically another 10 times in distilled water, from which aliquots were added to assay tubes. The dried milk samples were reconstituted and then treated in a similar manner.

The dried vegetable products were prepared by steaming 1 gm. in 70 ml. of 0.1 N H_2SO_4 for 30 minutes, followed by neutralizing with sodium acetate to pH 4.5. The material was diluted to 100 ml. and autoclaved for 15 minutes. 1 ml. of the sterile supernatant was diluted to 100 ml. in sterile distilled water from which aliquots were added to the assay tubes.

The assay values given agree favorably with those reported in the literature. Also there is a satisfactory agreement in most cases with those found on the same samples with the use of *Phycomyces blakesleeanus* as the test organism (the latter tests being made by Dr. K. C. Hamner).

The results reported were obtained by averaging the values from two or more levels run in duplicate. In no case was there found any consistent trend, either upward or downward, in the values at different levels. With few exceptions the assay values at different levels agreed within 15 per cent of one another. Occasionally, however, one finds a tube that gives a value entirely out of line with its duplicate, and those found at other levels, which emphasizes the necessity of running more than one level.

Recovery Experiments—Extracts of the three dried vegetable products mentioned in Table II were prepared as described above. Pure thiamine was added to parts of the extracts in amounts equal to 5 γ per gm. of the original food. The total thiamine content was determined in the controls, as well as in the samples with added thiamine, and the per cent recovery of added vitamin calculated. The results are shown in Table III.

The recovery experiments, as shown in Table III, indicate that in some food products there may be certain stimulating substances other than those present in the basal medium. For accurate quantitative determinations it may be desirable to add to the medium an extract of the specific food that has been freed of thiamine, either by fullers' earth adsorption or by sulfite treatment.

Chemical Specificity—Increasing concentrations of 4-methyl-5-β-hydroxyethylthiazole and 2-methyl-5-ethoxymethyl-6-aminopyrimidine, both alone and in combination, were added to the basal medium, ranging from 1 millimicrogram to 10 γ per 10 ml. of medium. The growth which occurred in all tubes was no greater than that found in the basal medium. Experiments with sulfite cleavage (2) with pure thiamine preparations, as well as with food products, consistently showed over 99 per cent destruction of the activity.

Additional tests on the chemical specificity revealed that cocarboxylase is approximately 40 per cent more active than thiamine, calculated on a molecular basis. This has been found to be true on repeated trials with

different thiamine preparations. The results cited in Table IV indicate that the increased activity of cocarboxylase cannot be explained on the basis of impurity of the thiamine preparations used.

A cocarboxylase solution containing 10 γ per ml. was made up in 0.1 M acetate buffer, pH 4.5. 5 ml. of this preparation were mixed with an equal amount of a 2 per cent taka-diastase solution in acetate buffer. The mixture was incubated at 45° for 6 hours, after which the enzyme was inactivated by steaming for 10 minutes. Solutions of thiamine and cocarboxy-

TABLE III

Recovery Experiments with Dried Vegetables

Material assayed	Thiamine added per gm. sample	Thiamine found per gm. sample	Recovery of added thiamine
	γ	γ	*per cent*
Whole wheat flour	0	6.6	100
	5	11.6	
Dried tomatoes	0	12.8	118
	5	18.7	
" turnip tops	0	4.2	124
	5	10.4	

TABLE IV

Comparative Activities of Thiamine and Cocarboxylase after Different Treatments

	Treatment	Activity
		per cent
Thiamine	Untreated	100
	N HCl, 100°, 30 min.	101
	Taka-diastase*	110
Cocarboxylase	Untreated	140
	0.1 N H_2SO_4, 121°, 20 min.	130
	N HCl, 100°, 30 min.	131
	Taka-diastase*	98

* 1 per cent taka-diastase, 45°, 6 hours.

lase of similar concentration were also treated by steaming for 30 minutes in N HCl, and autoclaving at 121° for 20 minutes in 0.1 N H_2SO_4. The activities of all treated solutions, compared to that of untreated thiamine, are shown in Table IV. Suitable controls were included to correct for the thiamine content of taka-diastase.

It can be seen that upon hydrolysis of the thiamine pyrophosphate molecule with the contaminating phosphatase in the enzyme preparation, the activity lowers to approximately the theoretical. Cocarboxylase treated with inactivated taka-diastase showed no decrease in activity.

Heating cocarboxylase in acid solutions, sufficient to hydrolyze 1 phosphoric acid molecule from the coenzyme (11), seems to lower the activity only to a slight degree.

It is not the purpose of this communication to discuss methods of preparation of samples for thiamine assay. However, from the data cited above it would seem necessary to treat food samples, known to contain large amounts of cocarboxylase, with a phosphatase preparation in order to avoid high results. Taka-diastase digestion has been reported to be desirable for the liberation of most of the B vitamins in food products (12).

Taka-diastase digestion of the three dried vegetable products listed in Table III resulted in no significant decrease of the determined thiamine content.

DISCUSSION

The proposed microbiological assay for thiamine appears to be superior to other procedures now in use. The test organism cannot utilize either or both cleavage products of the vitamin, which might occur in certain processed foods. Furthermore, cocarboxylase is active, but the determined assay levels may be slightly high unless suitable steps are taken for foods having relatively high concentrations of the coenzyme.

No explanation is offered for the greater activity of cocarboxylase. One might conclude that cocarboxylase is incorporated directly into the cell of *Streptococcus salivarius* without previous hydrolysis of the pyrophosphoric ester.

Because of the extreme sensitivity of the test organism toward thiamine no interference is encountered due to the turbidity of added food extracts. Milk or milk powder can be assayed with no difficulty when turbidimetric methods are used for measuring growth response.

When thiamine is totally excluded, no growth occurs in the basal medium. Attempts to train the test organism to synthesize its thiamine requirements have met with failure. Thiamine supplied in large excess has not been found to be inhibitory.

Although only one strain of *Streptococcus salivarius* was used throughout this study, it is believed that the assay procedure is not dependent upon one particular culture. Twenty different cultures of *Streptococcus salivarius* have been found to be identical with respect to their growth factor requirements and it is probable that any strain of this species would be equally satisfactory as a test organism in regard to specificity, sensitivity, and accuracy. These organisms can be easily detected and isolated by plating a throat smear on sucrose-gelatin agar as described elsewhere (8).

ethylthiazole, and 2-methyl-5-ethoxymethyl-6-aminopyrimidine, and to Dr. K. C. Hamner, United States Nutrition Laboratory, Cornell University, for assaying the food materials by the *Phycomyces blakesleeanus* method.

SUMMARY

A rapid and specific microbiological assay procedure for the determination of thiamine is proposed, based upon the growth response of *Streptococcus salivarius* to thiamine. The "thiazole" and "pyrimidine" moieties are not active under the conditions tested. Cocarboxylase is 40 per cent more active than thiamine, calculated on a molecular basis.

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THE EFFECTIVENESS OF A MIXTURE OF ARGININE, GLYCINE, AND CYSTINE IN THE PREVENTION OF THE SO CALLED VITAMIN B_4 DEFICIENCY IN THE CHICK*

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In 1933 Keenan, Kline, Elvehjem, Hart, and Halpin (1) described a deficiency condition in the chick resulting from a lack of an unknown factor found in liver. This condition was characterized by atrophy of the leg muscles, difficulty in locomotion, and lack of growth. Because this factor had properties similar to those of vitamin B_4 reported necessary for the rat by Reader (2), the Wisconsin workers adopted the name of vitamin B_4 for the chick factor. Since that time several reports on this factor have appeared from this laboratory, including work on its distribution (3) and methods of assay (4).

With the discovery of newer vitamins in the years following 1933 and with the improvement of experimental rations, it became evident that a material of vitamin nature, which answered the description of vitamin B_4 for the chick, could not exist. It was obvious, therefore, that one or more of the known vitamins or amino acids were responsible for the prevention of the deficiency symptoms.

A clue to the answer of this problem was found in the work of Hegsted, Briggs, Elvehjem, and Hart (5) which suggested that the chick vitamin B_4 might be identical with a mixture of arginine and glycine, since these amino acids, when fed to chicks on purified rations containing 18 per cent of casein, prevented the occurrence of symptoms characteristic of vitamin B_4 deficiency. Later it was demonstrated by Briggs, Mills, Elvehjem, and Hart (6) that the rations which were low in arginine and glycine were also low in cystine (or its equivalent of methionine). For this reason it was thought that the vitamin B_4 deficiency syndrome in the chick might be due to a lack of a mixture of all three of the amino acids. In order to answer the question completely it was necessary to go back to the original

vitamin B_4-low ration upon which the chick deficiency symptoms developed and to find whether these amino acids prevented this condition.

This paper presents the results obtained after the addition of various supplements to the vitamin B_4-low ration. It has been found that good growth is obtained and paralysis prevented with this ration when the combination of arginine, glycine, and cystine is fed.

EXPERIMENTAL

Every effort was made to make the experimental procedure as similar as possible to that which was outlined by Keenan *et al.* (1). The original ration (No. 441) consisted of dextrin 53.4, crude casein 24, Salts 40 2.5, yeast (Anheuser-Busch Strain C) 8, autoclaved liver residue 10, and cod liver oil 2. The liver residue was autoclaved for 5 hours at 15 pounds and this with the yeast was the source of the vitamin B complex. Day-old white Leghorn chicks were used and the experiment was conducted for 5 weeks. The experimental ration, made up every 2 weeks, and water were fed *ad libitum*.

Results

The results (Table I) show that chicks receiving the basal ration grew slowly (weighing 189 gm. at 5 weeks) and three out of the twelve chicks developed a vitamin B_4 paralysis as described by Bird and Oleson (7). Arginine, when fed alone, increased the amount of growth by approximately 60 gm. Glycine, alone, likewise caused some improvement in growth, but when arginine and glycine were fed together better growth than with either one alone was obtained. The paralysis seemed to be prevented by either one or both of the amino acids. When cystine was fed with arginine and glycine (Group 5) nearly maximum growth was obtained. These chicks weighed an average of 140 gm. more than the basal group and appeared normal in all respects. Slightly better growth was obtained by feeding a mixture of solubilized liver, known vitamins, and additional salts with the three amino acids (Group 6).

Since it had been demonstrated that a combination of arginine, glycine, and cystine (6) substituted for the "cartilage factor" (8), cartilage was fed to the chicks in Group 7. As would be expected marked improvement over the basal group was noted. Additional vitamins A and D and vitamin B_1 (Groups 8 to 10) gave no appreciable difference in growth of chicks in similar groups without these vitamins.

As to feathering (Table I), the amino acids arginine and glycine gave normal feathers, as demonstrated previously on other rations (5). Slight improvement in feathering over the basal ration was obtained with addi-

tional vitamins A and D. This may be explained by the work of Bird and Oleson (7), who reported that similar rations low in vitamin B_4 became rancid, thus favoring destruction of vitamin A.

The deficiency picture was further complicated by the fact that twelve chicks had encephalomalacia, as described by Pappenheimer and Goettsch (9), regardless of the supplement given. Encephalomalacia has been noted previously by Elvehjem, Phillips, and Hart in similar rations and has been differentiated from a vitamin B_4 deficiency (10).

TABLE I

Growth of Chicks Receiving Vitamin B_4-Deficient Ration with Various Supplements

Group No.	Supplement to basal Ration 441	No. of chicks	No. dead at 5 wks.	Weight at 5 wks.	No. with vitamin B_4 paralysis	Feathers at 5 wks.
				gm.		
1	None	12	2	189	3	Fair
2	0.5% arginine	6	1	250	0	Good
3	3% glycine	6	1	233	0	"
4	0.5% arginine + 3% glycine	6	0	264	0	Very good
5	0.5% " + 3% " + 0.3% cystine	6	0	329	0	" "
6	As Group 5 + 2% solubilized liver + 3% salts + fat-soluble and B complex vitamins	6	0	363	0	" "
7	10% cartilage	6	1	293	0	" "
8	Vitamins A and D (2 drops of a concentrate weekly)	6	0	176	1	Fair to good
9	0.5 mg. vitamin B_1 per 100 gm.	6	2	175	2	"
10	As Group 5 + 0.5 mg. vitamin B_1 per 100 gm.	6	0	340	0	Very good

DISCUSSION

The principal deficiency in the vitamin B_4-low ration is the amino acids arginine, glycine, and cystine. When these three amino acids are supplied, good growth occurs and the vitamin B_4 deficiency symptoms are prevented. Arnold, Kline, Elvehjem, and Hart first showed, in 1936, the importance of arginine in chick nutrition (11). These workers, using a vitamin B_4-low ration similar to ours, obtained a growth response with arginine and also reported that the chicks were helped in appearance with this amino acid. They made a distinction between arginine and vitamin B_4, however. The value of glycine has been demonstrated by Almquist, Stokstad, Mecchi, and Manning (12). Klose and Almquist reported the necessity of methi-

onine in purified chick rations (13).   The importance of a combination of
arginine, glycine, and cystine, when added to rations containing 18 per cent
of casein, has been shown previously (6).   The combination of these three
amino acids is important in the prevention of gizzard erosion (6), which may
explain in part the occurrence of gizzard erosion on vitamin B_4-deficient
rations (4).

It is interesting to note that Luckey *et al.* recently found that rations con-
sisting mainly of whole milk powder are low in arginine, glycine, and
cystine when fed to chicks (14).

Reader, in 1929 (15), obtained a deficiency in the rat due to a lack of a
factor found in certain concentrates of liver which was later termed vitamin
B_4 (2).   Since it has been amply demonstrated that rats can grow normally
on a diet containing all of the known vitamins, it is obvious that the rat
vitamin B_4 deficiency of Reader must, of consequence, be due to a lack of
one of the known vitamins or a combination of them.   Hence, it is not to be
implied that the results with the chick vitamin B_4 presented in this paper
apply to the rat.

Swank and Bessey, working with chronic thiamine deficiency in pigeons,
have raised the question whether or not vitamin B_4 deficiency in the chick is
due to a chronic vitamin B_1 deficiency (16).   Our results demonstrate that
this is not the case and further proof, in the line of histological evidence, will
be presented elsewhere.

SUMMARY

It has been found that a mixture of arginine, glycine, and cystine, when
fed in a vitamin B_4-low ration to chicks, promotes good growth and prevents
a typical paralysis.

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A NEUROSPORA ASSAY FOR PYRIDOXINE

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Of all the B complex vitamins available in pure form, the assay for pyridoxine (vitamin B_6) in complex biological materials has been least satisfactory. Animal assay methods for determining vitamin B_6 in biological materials are time-consuming and expensive. Existing chemical methods are unsatisfactory because of unknown interfering substances, numerous and varied manipulations of large volumes of solution, lack of sensitivity, etc. (1, 2), although Bina, Thomas, and Brown have overcome most of these difficulties in their recently proposed method (1). Microbiological procedures involving *Lactobacilli* which are satisfactory for the assay of most of the B vitamins have not been equally effective in the determination of pyridoxine owing primarily to the response of the *Lactobacilli* to pseudopyridoxine (3, 4). Vitamin B_6 values of meats and wheat products obtained by the yeast assay method of Williams, Eakin, and McMahan (5) are much lower than those of animal assays (6, 7). While the present *Neurospora* assay method for pyridoxine was being developed, another yeast method appeared (2) which yields pyridoxine values for various biological materials that agree moderately well in a number of instances with those of animal assays on similar materials.

In the present method, we have made use of an x-ray-induced mutant of the mold, *Neurospora sitophila*, produced by Beadle and Tatum (8), which requires added pyridoxine for growth except under very special conditions of pH and nitrogen nutrition (9). Those investigators suggested the use of the mutant for assay of vitamin B_6 and made some preliminary experiments in that direction.

The *Neurospora* assay method for pyridoxine, described below, is based on dry weight of the mold growth. In contrast to turbidimetric procedures, it can be readily applied to highly colored or turbid solutions. It is not influenced by pseudopyridoxine. Of most significance is the fact that *Neurospora* pyridoxine values of meats, wheat, flour, etc., are in good agreement with those of animal assays on the same or similar biological materials.

EXPERIMENTAL

Organism—The "pyridoxinless" mutant, No. 299, of *Neurospora sitophila* was used. We are greatly indebted to Dr. Beadle for supplying us

with this culture. Stock cultures are grown on Sabouraud agar slants (maltose 38 gm., Bacto-peptone 8 gm., Bacto-malt extract 2 gm., agar 20 gm., H_2O 1 liter) at 30°. Pink- or orange-colored spores usually are abundantly formed at the upper portion of the slant in 4 or 5 days. After sporulation, the cultures are stored in the refrigerator until used. Fresh cultures are prepared every 3 weeks.

Basal Medium—The composition of the basal medium is given in Table I. This formula is twice the desired final concentration. It is helpful to prepare stock solutions of the salts in concentrations convenient for pipetting. The medium may be opalescent but this does not interfere with the assay. The zinc represses sporulation, which facilitates harvesting of the mold. KH_2PO_4 buffers the medium initially at approximately pH 5. This is essential, since growth without pyridoxine will occur if the pH rises above 5.8 to 6.0 (9). Ammonium tartrate also aids in keeping the pH below the critical value, for as the NH_4 ion is utilized by the fungus tartaric acid accumulates in the medium and lowers the pH.

TABLE I

Medium for Pyridoxine Assay (Undiluted)

Sucrose	30.0 gm.	$CaCl_2$	0.2 gm.
NH_4 tartrate	10.0 "	$FeCl_3$	10.0 mg.
KH_2PO_4	5.0 "	$ZnSO_4 \cdot 7H_2O$	2.0 "
$MgSO_4 \cdot 7H_2O$	1.0 "	Biotin	8.0 γ
NaCl	0.2 "	Distilled H_2O	1 liter

Assay Procedure—5 cc. quantities of the basal medium are placed in 50 cc. Erlenmeyer flasks. 0.5, 1.0, 1.5, and 2.0 cc. amounts of the extract or solution of the sample prepared as described below are next added, in duplicate, and the total volume in each flask is made to 10 cc. with water. Similarly, a set of reference flasks is prepared containing from 0.1 to 1.0 γ of pyridoxine (the amounts refer to the free base and not the hydrochloride). Blanks containing no pyridoxine are included. The flasks are plugged with cotton and autoclaved at 15 pounds pressure for 15 minutes. After sterilization they are allowed to cool and are then inoculated with a spore suspension of *Neurospora sitophila*. The inoculum is prepared by removing with a platinum loop a small portion of growth, about 3 mm. in diameter and consisting primarily of spores, from an agar slant culture and making a uniform suspension in 10 cc. of sterile water. Each assay flask receives 1 drop of the spore suspension delivered from a 1 cc. pipette. Small variations in the number of spores inoculated do not affect the assay. A very light inoculum may prevent the standard curve from rising to its normal maximum growth level of 40 to 45 mg. of mycelium. This may

also occur if the culture is too old.   An unusually large inoculum may increase growth in the blanks to more than the customary 1 mg. of mycelium.   None of the above conditions invalidates the assay provided the weights of the standard or reference cultures form a uniformly ascending curve when plotted against micrograms of pyridoxine.

The inoculated flasks are incubated at 30° for 5 days, during which time a mat or pellicle of fungus growth develops on the surface of the medium if pyridoxine is present.   In a few assays the incubation period was reduced to 4 days or increased to 6 days without any harmful effect.   After incubation, the cultures are steamed at 100° for 5 minutes and the myeelium is harvested.   It is easily removed from the flask in one piece with a stiff wire needle.   Bits of mycelium that have grown up the sides of the flask are wiped up with the main portion of the pellicle.   The fungus growth is pressed dry between paper towels, rolled into a small pellet, and dried at 100° for 2 hours.   Glazed porcelain spot plates (112 × 92 mm.) are con-
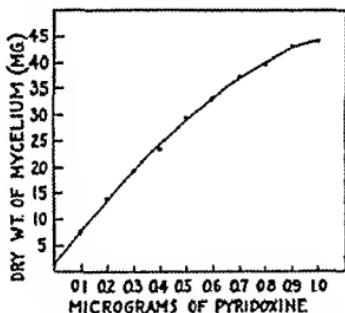
Fig. 1. Growth response of *Neurospora sitophila* to pyridoxine

venient for handling the mycelium during drying and weighing.   The pellets from duplicate flasks can be placed in a single depression of the spot plate and labeled with a heat-resistant type of china-marking pencil.   The dry mycelium is weighed to the nearest mg. on an analytical balance.

Growth response to increments of pyridoxine ranging from 0.1 to 1.0 γ is shown in Fig. 1.   A similar standard curve is made with each set of assays.   Dilutions of the material under test are chosen to give dry weight yields of the fungus which fall on the sharply ascending portion of the standard curve and their pyridoxine content is read from the curve.   The final value is an average of the figures obtained at the various levels which agree closely (Table II).

Preparation of Samples for Assay

Extraction of Pyridoxine—Insoluble materials are ground or finely chopped prior to extraction.   An appropriate aliquot of the sample, generally

1 to 5 gm., is mixed with 40 cc. of 1 N HCl[1] in a 250 cc. Erlenmeyer flask and autoclaved at 15 pounds pressure for 1 hour. This treatment should be adequate to liberate pyridoxine from soluble and insoluble complexes (2). It will also thoroughly digest insoluble substances so that even 15 per cent flour suspensions become completely fluid and easy to manipulate. If the sample is in solution after HCl extraction, it is cooled and diluted to 50 cc. with water. However, if an appreciable amount of insoluble material remains after extraction, the sample is filtered through paper while hot into a 50 cc. volumetric flask and the residue on the paper is washed twice with small quantities of water and the total filtrate is diluted to 50 cc. with water.

Destruction of Thiamine—Thiamine in the sample must be destroyed, since it stimulates growth of the test organism in the presence of sub-

TABLE II

Pyridoxine Content of Materials at Different Assay Levels

Liver concentrate			Yeast extract			Wheat		
Amount per assay flask	Pyridoxine		Amount per assay flask	Pyridoxine		Amount per assay flask	Pyridoxine	
	Found	Content		Found	Content		Found	Content
mg.	γ	*γ per gm.*	*mg.*	γ	*γ per gm.*	*mg.*	γ	*γ per gm.*
5	0.10	20	5	0.12	24	25	0.15	6.0*
10	0.17	17	10	0.23	23	50	0.20	4.0
15	0.27	18	15	0.35	23	75	0.28	3.7
20	0.38	19	20	0.40	20	100	0.37	3.7
Average.........		18.5			22.5			3.8

* Omitted from the average.

optimum amounts of pyridoxine (9). A modification of the sulfite cleavage method of Schultz, Atkin, and Frey (10) is used. 10 cc. of the extract are pipetted into a 50 cc. Erlenmeyer flask and 0.7 cc. of 10 N NaOH is added, followed by 5 cc. of a freshly prepared 1 per cent solution of anhydrous sodium sulfite. The pH of the mixture is adjusted to 8 with 1 N NaOH, cresol red being used as an outside indicator. The total amount of alkali added preferably should not exceed 2 to 3 cc. The cleavage of thiamine is carried out at pH 8 instead of pH 5 as recommended, because it was found that considerable destruction of pyridoxine occurs when solutions of pyridoxine plus thiamine are treated with sulfite at pH levels of less than 8. Also, the amount of sulfite used is one-half that suggested by Schultz *et al.* to avoid toxic effects encountered with larger quantities. However, it is

[1] Liquid samples which contain only small amounts of pyridoxine, for example milk, are made 1 N with concentrated HCl to avoid excessive dilution.

sufficient to destroy at least 100 γ of thiamine. After adjustment to pH 8, the mixture is steamed in the autoclave at 100° for 30 minutes, cooled, and treated with an exact quantity of a fresh 2 per cent H_2O_2 solution to destroy excess sulfite. Usually 5 to 10 drops of peroxide are required to reach the end-point.[2] The volume is made to 20 cc.[3] with water and the sample is assayed for pyridoxine as described above.

DISCUSSION

The *Neurospora* assay method for determining pyridoxine satisfies the usual criteria of reliability in that (a) assay values obtained from different dosage levels of test samples agree closely, indicating absence of stimulatory

TABLE III

Reproducibility of Assays

Material	Pyridoxine content			
	Assay 1	Assay 2	Assay 3	Mean
	γ per gm.	γ per gm.	γ per gm.	γ per gm.
Yeast extract (Bacto).......................	21.0	23.3	20.0	21.4
Corn steep liquor...........	9.8	10.0	9.6	9.8
Liver concentrate.........................	19.0	17.0	17.0	17.7
Wheat, whole.................	3.8	3.7	3.8	3.8
Flour, white.............................	2.6	3.0	2.7	2.8

or inhibitory substances (Table II); (b) there is excellent duplication of assay values on the same material in different experiments (Table III); (c) recoveries of pyridoxine added to biological materials are quantitative within the limits of experimental error, *viz.* ±15 per cent (Table IV); and (d) pyridoxine values obtained from meats, wheat, and wheat products agree closely with those of animal assays on the same or similar materials (Table V).

The basal medium, although completely synthetic, appears to be adequate for maximum growth of *Neurospora* when supplied with pyridoxine, since the addition of 0.2 per cent vitamin-free hydrolyzed casein does not increase growth of the fungus.

[2] "The end point of the reaction is determined with the aid of an outside indicator made by mixing one drop of 5 per cent potassium iodide, one drop of 1 per cent soluble starch indicator and one drop of 50 per cent sulfuric acid. This mixture develops a pink tinge which gets darker with time. If a drop of a sulfite-containing solution is transferred to this indicator by means of a glass rod, the pink color is discharged. As the end point of the titration is approached the pink color fails to disappear and when an excess of peroxide is present a deep violet color appears" (Schultz, Atkin, and Frey (10)).

[3] This represents a further 1:1 dilution of the sample to be considered in calculating its pyridoxine content.

Although thiamine can partially replace pyridoxine in the nutrition of the mutant strain of *Neurospora sitophila* and must, therefore, be excluded from pyridoxine assays, other growth factors such as nicotinic, pantothenic, and *p*-aminobenzoic acids, riboflavin, inositol, folic acid (concentrate),

TABLE IV

Recovery of Pyridoxine

Material	Pyridoxine content	Pyridoxine added	Total	Pyridoxine recovered	Recovery
	γ per gm.	γ per gm.	γ per gm.	γ per gm.	per cent
Liver concentrate..................	16.0	20.0	36.0	31.2	87
Yeast extract.................. ..	19.0	20.0	39.0	40.0	103
Corn steep liquor..................	9.4	10.0	19.4	16.1	83
Wheat..........................	3.8	5.0	8.8	7.9	90

TABLE V

Pyridoxine Content of Biological Materials

Material	Pyridoxine	Animal values in literature[*]
	γ per gm.	γ per gm.
Wheat, ground seed....................	3.7	3.8 (7)
Rye, ground seed	3.5	
Barley, ground seed....................	4.6	
Buckwheat, ground seed..............	3.5	
Millet, ground seed....................	3.3	
Wheat germ[†]..........................	14.8	11.3–14.6
Flour, patent..........	2.3	1.8 (7)
Beef, fresh.........................	2.8	3.1, 3.3 (6)
Veal, "	2.8	3.3, 3.4, 3.6 (6)
Lamb, "	3.6	2.5 (6)
Pork, "	3.3	3.7, 4.2, 5.3 (6)
" liver, fresh.....................	4.3	2.6, 2.7 (6)
Milk, whole, A........................ .	0.58 (per cc.)	1.1 (6)
" " B.................	0.58 " "	
Peptone, Armour..................	8.0	
Heart infusion broth, Bacto.......... .	11.3	
Malt extract, Bacto.............	5.4	
Beef " "	7.2	

* Expressed in terms of vitamin B_6 and not vitamin B_6 hydrochloride as recorded in the literature; obtained by multiplying the literature values by 0.82.

† This sample and its pyridoxine value by rat assay were kindly supplied by Dr. C. A. Elvehjem.

uracil, adenine, guanine, and xanthine do not stimulate the development of the fungus at suboptimum levels of pyridoxine.

It has been noted that with a few concentrates, such as peptone or malt extract, there is a tendency at the 1.5 cc. and 2.0 cc. assay levels for the

pH of the cultures to rise above the critical pH of 5.8. When this occurs, maximum growth of the fungus is obtained irrespective of the amount of pyridoxine present. It can be controlled by doubling the KH_2PO_4 content of the medium.

Unlike the *Lactobacilli*, the mutant strain does not respond to pseudo-pyridoxine. This is shown by the fact that autoclaving of solutions of pyridoxine with hydrolyzed casein does not increase the biological activity of pyridoxine as is the case with the bacteria. The good agreement of pyridoxine values of natural products obtained by *Neurospora* assay with those of rat assays is also evidence that the fungus is not influenced by pseudopyridoxine. *dl*-Alanine, which in sufficient concentration completely replaces vitamin B_6 for *Streptococcus lactis* (11), does not alter the pyridoxine requirements of *Neurospora*.

Scudi has reported the presence of a soluble conjugate of pyridoxine in water-soluble extracts of rice bran (12). For one of two samples of rice

TABLE VI

Effect of Extraction Procedure on Pyridoxine Assays

Material	Pyridoxine content on extraction* with		
	H_2O	0.1 N HCl	1 N HCl
	γ per gm.	γ per gm.	γ per gm.
Liver concentrate..........................	19	22	19
Rice bran concentrate.....................	40	55	130
Yeast extract..............................	24	25	21
Corn steep liquor.........................	9.0	9.4	9.8

* Autoclaved at 15 pounds for 1 hour.

bran concentrate he obtained pyridoxine values ranging from 41 to 49 γ per gm. before and 100 to 115 γ per gm. after acid hydrolysis. With the same sample of rice bran concentrate, kindly supplied by Dr. Scudi, *Neurospora* assays yielded 40 γ of pyridoxine per gm. on extraction with water and 130 γ of pyridoxine per gm. after hydrolysis with 1 N HCl (Table VI), which confirms Scudi's findings. However, acid hydrolysis of liver concentrate, yeast extract, and corn steep liquor, which are all water-soluble, did not increase their pyridoxine values as compared to water extraction. This may indicate that soluble bound forms of pyridoxine are not widely distributed, or that perhaps they had been hydrolyzed in the preparation of the concentrates.

SUMMARY

An accurate and specific microbiological method for the determination of pyridoxine has been developed, based upon the growth response to vitamin B_6 of a "pyridoxinless" mutant of the mold, *Neurospora sitophila*.

oxidation (6) and the formation of a soluble colored complex with excess dimethylglyoxime (7).

Reagents—

1. 10 per cent sodium tungstate and $\frac{2}{3}$ N sulfuric acid.

2. 50 per cent ferric chloride.

3. 10 N sulfuric acid.

4. 20 per cent sodium acetate.

5. 10 per cent hydroxylamine hydrochloride. This reagent keeps for at least 2 months in the refrigerator. "Neutral" hydroxylamine is prepared by neutralization of a portion of the stock with 6 N sodium hydroxide to approximately pH 7.4, phenol red being used as an inside indicator.

6. 0.5 per cent hydrated nickel chloride ($NiCl_2 \cdot 6H_2O$).

7. 0.02 N hydrochloric acid.

8. 0.1 per cent (by volume) bromine water.

9. 1.0 per cent dimethylglyoxime in 95 per cent alcohol.

10. Wash solution. This is an aqueous solution saturated with nickel dimethylglyoxime and octyl alcohol. The former is prepared by precipitation from nickel and dimethylglyoxime, recrystallized three times from dilute hydrochloric acid, and extensively washed with distilled water. A saturated solution of this substance is shaken with an excess of octyl alcohol, chilled in an ice bath, and filtered. The wash solution is conveniently kept in the refrigerator, since it is desired cold in the operation of the method.

11. Stock nickel standard: 0.2020 gm. of $NiCl_2 \cdot 6H_2O$ per liter; contains 50 γ of Ni per ml. Working standard: dilute 5 ml. of stock to 100 ml.; contains 2.5 γ of Ni per ml.

Acetoin Procedure—A 3 ml. sample of blood is delivered into 6 ml. of water, followed by 3 ml. of 10 per cent sodium tungstate and 3 ml. of $\frac{2}{3}$ N sulfuric acid. After complete mixing the tube is allowed to stand for 10 minutes, and then centrifuged for 15 to 20 minutes to obtain the maximum yield of filtrate. A clear tungstic acid filtrate from other tissues may also be used in the subsequent analytical steps.

A 7 ml. aliquot is transferred to a 25 ml. Pyrex test-tube to which are added 1 ml. of 10 N sulfuric acid and 2 ml. of 50 per cent ferric chloride. A clean rubber stopper is inserted, the contents mixed, and the tube placed in a boiling water bath for 15 minutes. If the stopper is inserted only lightly during the first several seconds in the bath in order to release excessive pressure, and the stopper then inserted firmly, no trouble is encountered with ejection of the stoppers.

A 9 ml. portion of the cooled solution is placed in the flask of the all-glass distilling unit described in a previous paper (8). The receiving vessel, placed in an ice bath, is a 15 ml. graduated Pyrex centrifuge tube containing 0.5 ml. of neutral hydroxylamine, 0.5 ml. of 20 per cent sodium acetate, and 0.2 ml. of 0.5 per cent nickel chloride. With the aid of a clean quartz pebble

in the flask and with the end of the condenser dipping into the nickel solution, distillate is collected to approximately the 4.5 ml. mark. The distillation should require approximately 2 minutes. The condenser is lifted from the distillate toward the completion of the distillation and approximately the last 0.5 ml. of distillate used for rinsing the inside of the condenser. A rubber stopper is inserted in the tube, the contents mixed by rotation, and the tube placed in a water bath maintained at 55–60° for $\frac{1}{2}$ hour. It is then cooled and allowed to stand in the refrigerator for at least 12 hours without shaking in order to obtain the heaviest crystals of nickel dimethylglyoxime. If no crystals are obtained, less than 5 γ of acetoin was originally present.

5 ml. of wash solution are added to the tube before centrifuging with simultaneous shaking to "wet" any floating or creeping precipitate. The octyl alcohol in the wash solution is quite effective in accomplishing this. The tube is centrifuged for 5 minutes and again shaken gently to submerge any traces of floating precipitate and loosen from the walls the less firmly adhering precipitate. After recentrifugation, the supernatant fluid is withdrawn to at least the 0.5 ml. mark by insertion of a capillary tube and suction. 10 ml. of the wash solution are then allowed to run down the side of the tube without disturbing the precipitate. It is again centrifuged, the supernatant fluid removed, and the washing procedure repeated once more.

2 ml. of 0.02 N hydrochloric acid are added to the washed precipitate and the remaining small volume of wash solution. The amount of nickel present in the remaining wash solution is entirely negligible. The precipitate is suspended by shaking and the tube placed in a boiling water bath. It is usually necessary to employ a stirring rod to loosen any precipitate adhering to the walls. When the precipitate is completely dissolved, 0.6 ml. of bromine water is added to the hot solution. If this is decolorized, another 0.4 ml. or more may be added until some color persists for at least $\frac{1}{2}$ minute while the tube remains in the bath. After cooling, 2 to 3 drops of concentrated ammonia are added to decolorize the bromine and the solution diluted to approximately 5 ml. 1 ml. of 1 per cent alcoholic dimethylglyoxime is added by forcible ejection from the pipette, the exact amount not being essential but the rapid mixing being necessary to avoid formation of a precipitate. Distilled water is added to the 10 ml. mark and the contents mixed. The color intensity of the resulting clear solution is measured in a photoelectric colorimeter or spectrophotometer at $\lambda = 460$ mμ, with water as a reference solution.

A standard is prepared by treating 2 ml. of the nickel working standard (5 γ of Ni) with bromine, the bromine decolorized, and the color developed as described above. The color is stable for at least 2 hours and its intensity very reproducible, so that it is only occasionally necessary to run a standard.

Diacetyl Procedure—Diacetyl can be distilled directly from a tube

acid filtrate of blood or tissues into the hydroxylamine-nickel mixture. The remainder of the determination is the same as described for acetoin.

If both acetoin and diacetyl are present, both are measured by the acetoin procedure; diacetyl can be determined separately by direct distillation, and the acetoin by difference from the total after ferric chloride treatment and distillation.

Calculations—1 γ of nickel is equivalent to 3.07 γ of acetoin or 2.93 γ of diacetyl. These factors may be employed directly or used to construct charts relating the color intensity to the amount of acetoin or diacetyl.

Testing of Method. Colorimetric Reaction for Nickel—The color reaction described for nickel depends on oxidation by bromine to tetravalent nickel and formation of an orange-red complex with dimethylglyoxime. A microgram of nickel is readily detected and 10 γ produce an intense color. The color proved to be stable and suitable for quantitative use. Amounts of

Fig. 1. The relation of color intensity to the amount of nickel. $\lambda = 460$ mμ

nickel ranging from 1 to 15 γ, either as the sulfate or chloride, were treated with bromine, ammonia, and dimethylglyoxime as previously described, and the color intensities read in the Coleman spectrophotometer at 460 mμ, with water as a reference solution. The results are plotted in Fig. 1 and show that there is a straight line relation between the amount of nickel and the log of the light absorption.

The intensity of the color was found to be considerably decreased by salts; so that it was desirable to minimize the concentration of hydrochloric acid used to dissolve the nickel dimethylglyoxime. The amount used did not change the color intensities from those recorded in Fig. 1.

Recovery of Nickel from Nickel Dimethylglyoxime—The liberation of nickel from nickel dimethylglyoxime was tested. The pure substance, prepared as previously described, was dissolved in warm 0.02 N hydrochloric acid and diluted so that 2 ml. portions contained 15 to 75 γ of the nickel complex.

These samples were treated successively with bromine, ammonia, and dimethylglyoxime as described under "Acetoin procedure." The results are recorded in Table I and indicate essentially complete liberation of nickel from nickel dimethylglyoxime.

Precipitation of Nickel Dimethylglyoxime—The precipitation of the nickel complex was studied at different pH values. Optimum precipitation occurred in approximately 2 per cent sodium acetate. Addition of acetic acid decreased the yield and addition of alkali led to the danger of precipitation of nickel hydroxide.

TABLE I

Recovery of Nickel from Nickel Dimethylglyoxime

Experiment No.	Ni dimethylglyoxime	Ni equivalent	Ni found	Recovery
	γ	γ	γ	*per cent*
1	15.0	3.05	2.95	97
2	30.0	6.10	6.00	98
3	45.0	9.15	9.05	99
4	60.0	12.20	12.30	101
5	75.0	15.25	14.95	98

TABLE II

Precipitation of Nickel Dimethylglyoxime

Experiment No.	Dimethylglyoxime	Ni equivalent	Ni found	Recovery
	γ	γ	γ	*per cent*
1	5.0	1.27	1.15	91
2	10.0	2.53	2.35	93
3	15.0	3.80	3.50	92
4	20.0	5.06	4.75	94
5	25.0	6.33	5.90	93
6	30.0	7.60	7.00	92

A solution of dimethylglyoxime containing 100 mg. per liter was prepared by first dissolving the solid in 10 ml. of 50 per cent alcohol before dilution with distilled water. Solutions containing 5 to 30 γ of dimethylglyoxime in 2 per cent sodium acetate and in a total volume of 5 ml. of solution were treated with 0.2 ml. of 0.5 per cent nickel chloride, and the nickel complex crystallized as previously described. The results are recorded in Table II and indicate a recovery of 91 to 94 per cent of the dimethylglyoxime.

Recovery of Acetoin and Diacetyl—Acetoin was purified by the ether treatment method of Stahly and Werkman (4). A standard solution was pre-

acid filtrate of blood or tissues into the hydroxylamine-nickel mixture. The remainder of the determination is the same as described for acetoin.

If both acetoin and diacetyl are present, both are measured by the acetoin procedure; diacetyl can be determined separately by direct distillation, and the acetoin by difference from the total after ferric chloride treatment and distillation.

Calculations—1 γ of nickel is equivalent to 3.07 γ of acetoin or 2.93 γ of diacetyl. These factors may be employed directly or used to construct charts relating the color intensity to the amount of acetoin or diacetyl.

Testing of Method. Colorimetric Reaction for Nickel—The color reaction described for nickel depends on oxidation by bromine to tetravalent nickel and formation of an orange-red complex with dimethylglyoxime. A microgram of nickel is readily detected and 10 γ produce an intense color. The color proved to be stable and suitable for quantitative use. Amounts of

FIG. 1. The relation of color intensity to the amount of nickel. $\lambda = 460 \, m\mu$

nickel ranging from 1 to 15 γ, either as the sulfate or chloride, were treated with bromine, ammonia, and dimethylglyoxime as previously described, and the color intensities read in the Coleman spectrophotometer at 460 $m\mu$, with water as a reference solution. The results are plotted in Fig. 1 and show that there is a straight line relation between the amount of nickel and the log of the light absorption.

The intensity of the color was found to be considerably decreased by salts; so that it was desirable to minimize the concentration of hydrochloric acid used to dissolve the nickel dimethylglyoxime. The amount used did not change the color intensities from those recorded in Fig. 1.

Recovery of Nickel from Nickel Dimethylglyoxime—The liberation of nickel from nickel dimethylglyoxime was tested. The pure substance, prepared as previously described, was dissolved in warm 0.02 N hydrochloric acid and diluted so that 2 ml. portions contained 15 to 75 γ of the nickel complex.

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IDENTIFICATION OF SMALL AMOUNTS OF ORGANIC COMPOUNDS BY DISTRIBUTION STUDIES. APPLICATION TO ATABRINE

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The best conventional micro technique of the present day chemist permits identification and establishment of purity for an organic compound when there is at hand an amount of the substance which is of the order of a few mg. A minimum of this much substance is required for an analysis and the determination of physical constants, such as melting point, boiling point, refractive index, etc., while final identification by the preparation of derivatives frequently requires a few mg. more of the substance.

Although experience has shown such a procedure in most cases to be the only unequivocal method of identification and establishment of purity, even the few mg. so required are still much too large a quantity to make feasible the study of the metabolic fate of many of our most active drugs when used in the therapeutic dosage. Such studies will require methods of detection, separation, and identification of the substance or its transformation products in amounts which will become ever smaller and smaller as the study proceeds and the fate of the drug is traced.

Thus the above limitations at once became apparent when certain proposed studies on the metabolic fate of the antimalarial drug atabrine were under consideration. At the present time, no reliable information exists in the literature, as far as we are aware, as to whether the drug, once it enters the blood stream, remains and exerts its effect on the malaria parasite as the unchanged drug, or whether, after all, it is some transformation product of atabrine which suppresses the growth of the parasite. Nor is information available as to whether atabrine is excreted unchanged in either the feces or urine, or whether transformation or detoxification products only are excreted. Accordingly, as part of a broader study of the chemistry, pharmacology, and medicinal use of the drug being carried on by other workers, it appeared desirable to attempt the development of suitable micromethods of detection and identification.

The present methods of detection and estimation of atabrine rely principally on colorimetric or fluorometric procedures, the latter being the more sensitive with the present day fluorescent photometer. Although these methods are admirable as far as sensitivity is concerned, the question of specificity at once presents itself, for these spectroscopic properties are due

chiefly to the acridine ring system, and small changes in the structure of the side chain or of the substituents on the ring might or might not be discovered. The total fluorescence or color should best be referred to as due to total acridines unless independent evidence is presented that only atabrine is responsible for the color or fluorescence. Nor would the quantitative aspects of the determination have real meaning unless they were due only to atabrine, for an unknown transformation product might have several times the intensity of color or fluorescence which atabrine possesses.

A suggestion by Dr. M. T. Bush, of Vanderbilt University School of Medicine, that the distribution coefficient of the drug between two immiscible liquids be determined as a physical constant for use in identification, served as the starting point for the development of a general procedure which may well prove useful in the rapid estimation of identity and, to a considerable degree, an estimation of purity as well. It was designed for use primarily when the total amount of material at hand is of the order of micrograms (20 to 100 γ) rather than of mg., but is not necessarily limited to such cases.

EXPERIMENTAL

The general procedure which at present appears most applicable to the atabrine problem is the following. 25 cc. of an ethylene dichloride solution of atabrine, which contains a known total amount (20 to 100 γ) and whose fluorescence is determined, are placed in a centrifuge separatory funnel, and 20 cc. of a cacodylic acid buffer solution are added. The mixture is well shaken and adjusted to a temperature of 24° ($\pm$1°). It is then centrifuged to clear the two layers, and sufficient of the top or aqueous layer is withdrawn by means of a pipette for the fluorescence determination. The lower layer is withdrawn through the stop-cock of the separatory funnel and its fluorescence likewise determined. The weight in micrograms per cc. of atabrine for the non-aqueous solution is then obtained by referring to a concentration-fluorescence curve previously determined experimentally with pure atabrine in ethylene dichloride solutions, and the fluorescence noted for the aqueous layer is converted to an arbitrary weight per cc. basis by referring it also to the ethylene dichloride-atabrine curve. *The ratio of the weight per cc. in ethylene dichloride solution to the apparent weight so derived for the aqueous layer then gives a figure which, for purposes of discussion here, will be called the apparent distribution constant, K_a.*

Both solutions are then returned to the separatory funnel as quantitatively as possible, and 5 cc. of methyl alcohol are added. The above procedure is then repeated and another apparent distribution constant obtained, which is characteristic for the particular dilution. This procedure is repeated, with progressive dilution by recorded volumes of methyl alcohol

until the two phases become mutually soluble (approximately 80 per cent methyl alcohol for the aqueous phase).

Thus a series of apparent distribution constants is obtained which will furnish a characteristic curve when the apparent distribution constants are plotted against the volume percentage of water in the aqueous phase. This percentage is arbitrarily based on the total amounts of methyl alcohol and water added, since the amount of methyl alcohol dissolved in the ethylene dichloride layer is unknown. Such a curve, determined on pure atabrine base, is shown in Fig. 1, Curve 2.
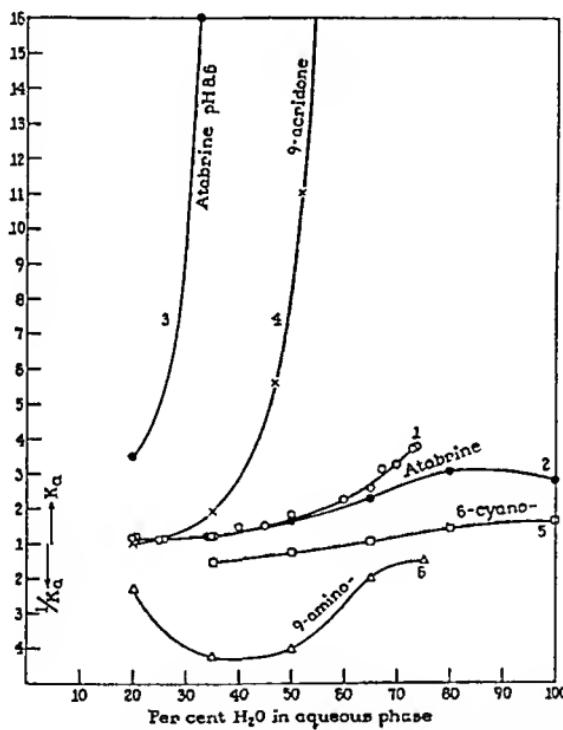
FIG. 1. Curves for the apparent distribution constants

An alternate procedure would be to add to the separatory funnel containing the ethylene dichloride solution of atabrine 20 cc. of methyl alcohol plus 5 cc. of the buffer. The dilutions would then be made by addition of recorded volumes of buffer. The curve so derived is shown in Fig. 1, Curve 1. This curve does not correspond with Curve 2 for reasons discussed below, but either curve should be equally characteristic of atabrine.

The values were found by experimentation to be readily reproducible, and theoretically should be characteristic of the pure substance. Any other compound studied in its place should give different apparent distribution constants, at least in one or more of the dilutions, and would thus result in

a curve which would be appreciably different. In support of this belief, several compounds closely related to atabrine were studied, and all were found to give entirely different curves.

The compounds used for this purpose were 2-methoxy-6-chloro-9-amino-acridine, Fig. 1, Curve 6, 2-methoxy-6-chloroacridone-9, Fig. 1, Curve 4, and 2-methoxy-6-cyano-9(4-diethylamino-1-methylbutyl)-aminoacridine, Fig. 1, Curve 5. These curves were determined by referring the fluorescence of their solutions back to the standard atabrine weight-fluorescence curve, and they do not therefore represent actual distributions.

Although the curves of these substances can readily be distinguished from that of atabrine, the question might be raised as to the type of curve to be expected when mixtures are encountered, as would probably be the
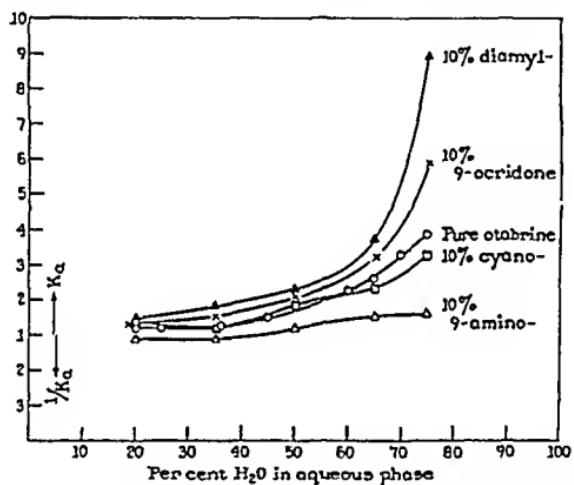
FIG. 2. Curves for the apparent distribution constants of synthetic mixtures

case when metabolic changes are being traced. Accordingly, mixtures of the above substances with atabrine were studied. Fig. 2 shows the curves obtained when mixtures containing 90 per cent atabrine + 10 per cent by weight of each of the above were taken. In addition, the curve from a mixture containing 10 per cent of 2-methoxy-6-chloro-9(3-diamyl-aminopropyl)-aminoacridine + 90 per cent atabrine is shown.

As a final check on the method, a known amount of atabrine was added to dog urine and prepared for determination by extraction with ethylene dichloride. The ethylene dichloride extract was then shaken with 5 per cent hydrochloric acid and, after separation, the acid layer was basified with sodium hydroxide. The basic solution was extracted with ethylene dichloride and, after being cleared in the centrifuge, the extract was used directly for the determination. The curve so obtained coincided within the experimental error with the standard curve.

Materials—The fluorometric determinations were made with a Pfaltz and Bauer fluorophotometer. The activating light (Hg lamp) passed through a filter which removed all but that of wave-length between 3000 and 4000 Å. The fluorescent light passed through a filter which removed any reflected ultraviolet light and the major part of the blue light, before it reached the recording cell.

A heavy fluorescent glass plate was used as the standard, and all measurements were corrected to this standard. Temperatures of the solution just at the time of making the measurements were recorded and when necessary adjusted to the proper range. The temperature of the fluorescing solution was found to have a considerable effect on the fluorescence; a lower temperature increased the fluorescence.

The standard buffer was a solution of 0.001 N cacodylic acid half neutralized with sodium hydroxide.

All solvents were examined prior to use for any fluorescence. The ethylene dichloride showed a very slight fluorescence, but this was not large enough to interfere seriously, although purification would be necessary if the method were extended to smaller amounts.

125 cc. separatory funnels, designed for use with the standard equipment of International centrifuge No. 2, were employed. The use of grease for the stop-cocks was avoided.

DISCUSSION

A discussion of the various factors which influence the experimental values found will perhaps make the procedure a more understandable one.

In the first place, the apparent distribution constants and the curve as well depend on the true distribution coefficients of the un-ionized base.

Secondly, the values of K_a depend on the intensity of the fluorescent light for any given intensity or wave-length of the activating light source. Such a fluorescent intensity is characteristic of the compound and is influenced greatly by the environment of the compound. For instance, the solvent used may exert a quenching effect, or the presence in the solvent of another compound in slight amount may produce quenching. Thus the relative change in intensity of fluorescence in going from one solvent to another could be in itself characteristic of the substance, and is inseparably incorporated as one of the contributing factors in the above curve of the apparent distribution constants.

Thirdly, the proportion of un-ionized base to the total responsible for the observed fluorescence in the aqueous phase depends on the mass law and on the pH of the solution. Thus the dissociation constant, a very characteristic physical property of organic bases, is also incorporated as one of the factors in the apparent distribution constants experimentally deter-

mined. The pH of the solution can readily be held constant by the use of a suitable buffer.

At the outset, it became apparent that cacodylic acid buffer had a marked quenching effect, since the total amount found in the aqueous (when its fluorescence is referred to the ethylene dichloride curve) and non-aqueous phases should otherwise equal the total amount taken at the beginning, but instead was found experimentally to be much less. Thus in the determination of the 100 per cent point value of the K_a curve, a total of 78 γ of atabrine was taken and, after equilibration and estimation, a total of 30.2 γ was found in the ethylene dichloride layer, while only 8.8 γ were found in the aqueous layer. A total of only 39 γ was accordingly accounted for in both layers by this procedure. The buffer was found upon investigation to be slightly soluble in the ethylene dichloride layer. Further indication that the fluorescence in the ethylene dichloride layer was not influenced by the cacodylic acid, if an appreciable amount were dissolved under these conditions, was obtained by the fact that a sample of ethylene dichloride which had been extracted with fresh buffer did not cause a quenching effect when used for the dilution of a known standard solution of atabrine.

If it is assumed that the ethylene dichloride layer is unaffected, the amount which actually is present in the aqueous layer can be obtained by difference from the total amount employed. Thus $78 - 30.2 = 47.8 \, \gamma$, compared to 8.8 γ observed. The discrepancy must be mainly ascribed to the quenching effect of the buffer, but may in small part also be due to the fact that the observed fluorescence is referred to the standard concentration-fluorescence curve of the base in ethylene dichloride instead of in water. In the determination of the apparent distribution constants, this procedure is arbitrarily used, since the solvent in the aqueous phase is undergoing progressive change.

Should the concentration present in the aqueous phase be obtained by difference, as outlined above, distribution constants are derived which more nearly approximate the true distribution coefficients on an actual weight basis, and a curve can be obtained which may have an entirely different shape for a substance which shows quenching in the aqueous phase. *For purposes of discussion in this paper, such distribution constants may be referred to as calculated distribution coefficients, or K_c.* However, even these may show some variation from the actual distribution coefficients of the base, since the presence of methyl alcohol dissolved in the ethylene dichloride has been disregarded, and this could influence to a certain degree the fluorescent estimation of the atabrine present. The relation of K_c to dilution of the aqueous phase for the base atabrine is shown in Fig. 3. The K_c curves for the 10 per cent mixtures previously mentioned are shown also.

The dissociation constants for atabrine have been determined by Christophers,[1] and are recorded (Christophers' terminology) as $pK_1 = 3.88$, and $pK_2 = 6.47$, respectively. From these, the relative proportion of ionized to un-ionized base in the aqueous phase can be calculated readily, since the hydrogen ion concentration is held constant at pH 6.2 by the cacodylic acid buffer. Thus

$$\frac{(BOH)}{(B^+)} = \frac{(OH)}{K} = 1.2 \times 10^{-4} \text{ (calculated from } pK_1)$$

If the calculated distribution constant, K_c, which has an actual weight basis and is not an arbitrary figure, as is K_a, be accepted as approximately correct, then it is possible to calculate the actual distribution coefficient
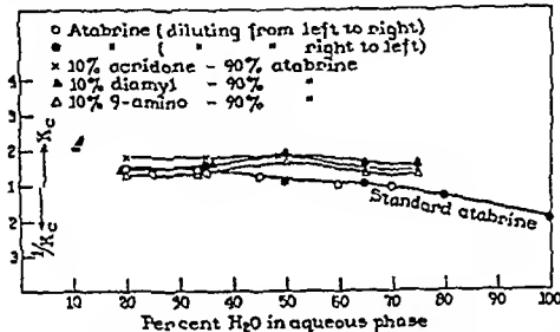
FIG. 3. Curves for the calculated distribution constants

for ethylene dichloride-water (buffered at 6.2) for the un-ionized base, although it must still be considered an approximation. Thus

$$\frac{C_{(BOH)_{ED}}}{C_{(BOH)_{H_2O}} + C_{(B^+)_{H_2O}}} = 2 \text{ (observed)}$$

and

$$C_{(B^+)_{H_2O}} = \frac{C_{(BOH)_{H_2O}}}{1.2 \times 10^{-4}}$$

therefore

$$\frac{C_{(BOH)_{ED}}}{C_{(BOH)_{H_2O}}} = 16,600 = K$$

Barring association in either of the phases, true distribution coefficients for such dilute solutions are independent of the amount of substance taken or volume of solvent, since otherwise Henry's law would not hold. It would thus appear at the outset that adjustment of the concentration of the

[1] Christophers, S. R., *Ann. Trop. Med.*, **31**, 43 (1937).

starting solution to a standard amount would not be necessary. However, in the curve for apparent distribution constants, which for purposes of characterization may be even more sensitive than the other, K_c, true distribution coefficients are not measured, since there is a large quenching effect involved and concentration can play an important rôle. It would be expected, therefore, that the K_a curve could be duplicated (for the case when quenching occurs) only when approximately the same amount of the pure material is used at the beginning of the determination. This was found to be the case with atabrine. On the other hand, the calculated distribution constant, K_c, should be independent of concentration and in fact proved to be so experimentally, within the ranges studied here.

For the study of an unknown solution, it is accordingly desirable to adjust the concentration by dilution until it is fluorometrically approximately the same as that used in the preparation of the standard curves with the pure drug.

It is for this reason that the alternate experimental procedure given above, while just as characteristic, does not give the same curve (Fig. 1, Curves 1 and 2) as that first outlined. However, calculation of the K_c values from Curve 1 resulted in figures (Fig. 3) which were in satisfactory agreement with those obtained when the dilution was made with methyl alcohol and the beginning made at the 100 per cent point.

The general method outlined is capable of many variations and may be extended to a variety of solvents. In the earlier stages of the study, ligroin was chosen as one of the solvents, but atabrine fluoresces much less in this solvent than in ethylene dichloride or chloroform. A larger amount of material is therefore necessary. Chloroform appeared to be just as suitable in the preliminary study as was ethylene dichloride.

Likewise, another buffer could be chosen. However, examination of the curves (Fig. 1, Curves 1 and 3) will show that the pH should not vary much from 6.2 in order to obtain the greatest sensitivity. Otherwise, the experimental error might be multiplied greatly, since the coefficients are ratios of concentrations. For instance, Curve 3, Fig. 1, was obtained with veronal buffer having a pH of 8.6. Only a small part of this curve could be studied with any degree of accuracy. Cacodylic acid buffer appears to be particularly suitable, for it is appreciably soluble only in the aqueous phase. Moreover, it appears to show a characteristic quenching with atabrine, but not with the other substances studied.

Since the method is essentially an analytical procedure, it is interesting to speculate in regard to the variation or experimental error one would expect in attempting to duplicate the curves given here for the pure base of atabrine. In this connection, the first and largest variable to be considered is probably the basic fluorometric analysis. The amount of this variation

or experimental error can only be learned by experience with the particular instrument employed. If for purposes of discussion it is granted that a 5 per cent experimental error in the galvanometer deflection might be expected, then the error such a 5 per cent deviation would cause in the value of K_a at the particular point in the curve would be of the order of ± 0.2. Experience appeared to show that the deviation encountered from experiment to experiment was occasionally somewhat larger than this figure with the technique employed here, as the divergence in the points on the curves indicates. Control of the temperature during the fluorometric determination to $\pm 1°$ proved to be adequate for the purpose, since a $1°$ change in the temperature of the fluorescing solution experimentally appeared to result in a deviation of approximately 3 per cent.

In order to keep the experimental error within the limits discussed above, it can be calculated readily on theoretical grounds that the buffer must not vary from the pH value of 6.2 by more than ± 0.2 pH unit. Conversely, it can be calculated that if the base were so altered that its dissociation constant would be changed by more than ± 0.2 from the experimentally determined value of pK_1, and all other factors, such as distribution, fluorescence, etc., remained the same, it would cause more than the allowable experimental error, and a transformation product would be indicated.

It seems wise to point out that much reliance should not be placed on the absolute values represented in curves such as are given here. Consequently, it should be emphasized that when an attempt is made to identify a substance a standard curve on the pure substance from another source and thought to be present should be run at the same time, with the same solvents, and under exactly the same conditions. In this way, certain unknown variables, such as a possible shift in the quenching influence of different preparations of the same solvent, may be canceled out and a much more sensitive comparison made. The individual curves given in this paper are, therefore, to be regarded as approximations, of value, perhaps, only in relation to the others, which were determined under identical conditions.

Finally, it should be understood that no claim is made in the present proposed method of identification for the achievement of the degree of reliability so well proved by the conventional procedures of organic chemistry. However, it is only fair to mention the fact that a fluorescent transformation product, in order to escape detection, must simulate the properties of atabrine to a rather improbable degree. In the first place, it must have quantitative solubility properties in several solvents, which are almost identical with that of atabrine, since otherwise it would not give the proper distribution constants. Secondly, it must possess nearly identical fluores-

cent properties and exhibit the phenomenon of quenching in a nearly identical way. Thirdly, it must possess a dissociation constant which does not vary from that of atabrine by more than 0.2 pK unit.

It is probable that the method could be made suitable for use with much less material than was here employed, if it should become necessary. With the apparatus at hand (15 cc. cuvettes), a complete curve could be run on 20 to 30 γ of atabrine without sacrificing appreciable sensitivity. The same result might be accomplished equally well on one-tenth or less of the amount either by using a smaller cuvette in the determination of fluorescence, or by employing a more sensitive fluorophotometer.

Experiments on Biological Fluids

A number of ethylene dichloride extracts, from blood or urine from patients and dogs which were receiving atabrine, was supplied by Dr. B. Brodie of the Goldwater Memorial Hospital. The blood or urine had been treated with alkaline buffer and directly extracted with ethylene dichloride. These extracts were then examined for quantitative fluorescence (in terms of atabrine) and extracted with 20 cc. of 5 per cent hydrochloric acid. The ethylene dichloride layer was then extracted with two successive 10 cc. portions of 5 per cent hydrochloric acid. Each of the acid layers was extracted successively with two 20 cc. portions of fresh ethylene dichloride. The ethylene dichloride layers were combined and examined for quantitative fluorescence. This may be designated the neutral fraction.

The acid layers were combined and made alkaline with a slight excess of 10 per cent sodium hydroxide solution. 25 cc. of ethylene dichloride were added and after shaking were separated by centrifugation. The ethylene dichloride layer containing the basic or atabrine fraction was then used directly for the distribution study. If it contained too large an amount of atabrine, it was adjusted by the proper dilution.

The alkaline layer which contained the acidic fluorescent material, and also the material which might be both acidic or phenolic and basic, was also examined for quantitative fluorescence.

Extracts from the blood of three patients receiving atabrine were studied. Nearly all the fluorescence appeared in the basic or atabrine fraction and only a small amount in the other fractions. When the basic fraction was studied by the distribution method, the results shown in Fig. 4 were obtained. None of the human blood extracts showed much divergence from the theoretical K_a or K_c curve. In two of the samples, this divergence was practically within the experimental error for the K_a curve, and was in excellent agreement for the K_c curve. The third (Jackson) showed an appreciable variation in both curves. It seems unlikely, however, that the amount of impurity could be very large. This was supported by the fact

that, at a later time, blood from three other patients receiving atabrine was investigated in the same way, except that here the extract was given an additional washing with 10 per cent NaOH. These extracts now corresponded well with the standard atabrine curve. Dog blood, on the other hand, showed more decided variation in both K_a and K_c curves, which appears to indicate one or more definite transformation products present.

Extracts from two human urines were investigated. Here again the majority of the fluorescence emerged in the basic fraction. Both of these showed a decided divergence from the theoretical atabrine curves (Fig. 5), and it must be concluded, therefore, that transformation products were present. In neither the K_a nor the K_c curves was the variation quantita-
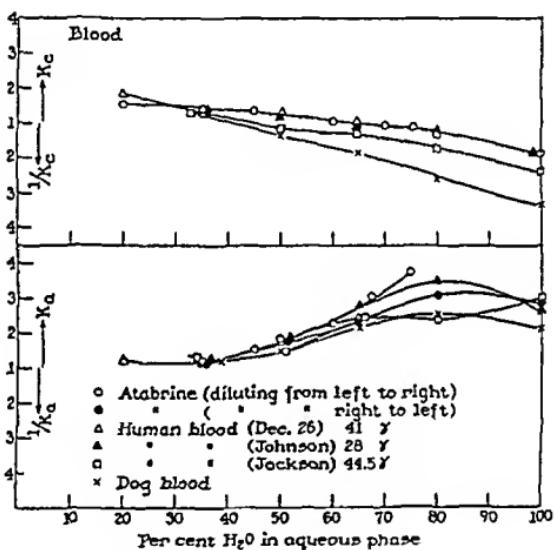
FIG. 4. Studies on blood

tively the same. Moreover, in the K_c curve, the divergence was not even in the same direction. From this behavior, it might be deduced that a different transformation product is present in one than in the other or, more probably, that both samples have at least two transformation products which are present in different relative proportions.

The results of the investigation of the extract of a dog's urine are shown in Fig. 6. The basic extract showed a definite divergence in both K_a and K_c curves. However, when the basic extract (atabrine fraction) was washed with 10 per cent NaOH until significant fluorescence was not present in the alkaline layer, the ethylene dichloride layer agreed well with the theoretical curves for atabrine. The manner in which the apparent impurity was removed suggested that it might be of phenolic character and
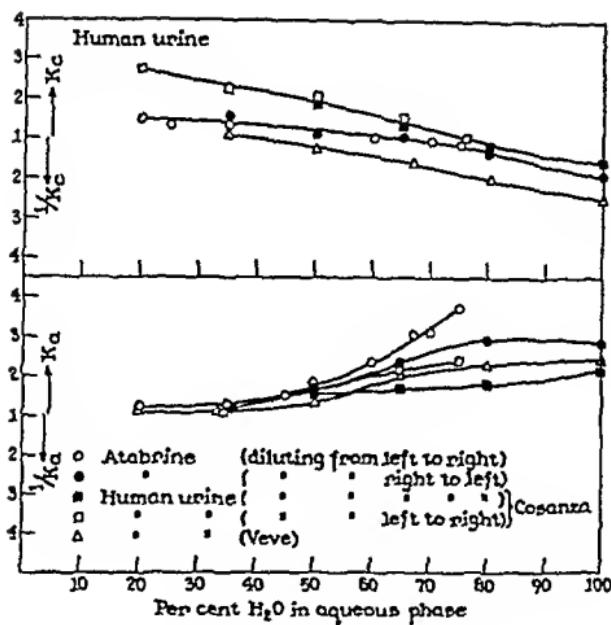
FIG. 5. Studies on urine
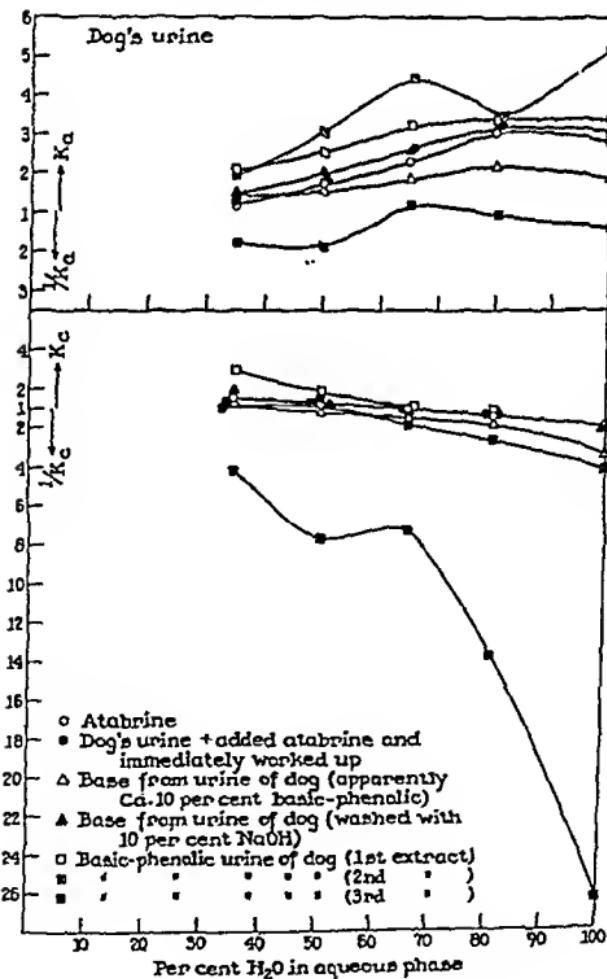
FIG. 6. Studies on dog urine

that the acidic or phenolic properties were not pronounced, but perhaps rather weak.

This view was further supported by the fact that a considerable fluorescence was also present in the phenolic or acidic-basic fraction of the preliminary separation. When this aqueous alkaline solution was extracted with fresh ethylene dichloride, the major part of its fluorescence was removed. The distribution behavior of such an ethylene dichloride extract is shown in Fig. 6, where it is designated as the first extract.

The alkaline layer from this extract was then extracted with fresh ethylene dichloride and a second extract so obtained. Its distribution behavior is also given in Fig. 6 (second extract). Again a major part of the remaining fluorescence was removed from the alkaline solution.

Finally, a third extract was made with fresh ethylene dichloride, and a small fluorescence was obtained. This third extract was also studied by the distribution method and the results likewise are shown.

From the divergence of all these curves, it can be deduced that a degree of fractionation has been realized by the progressive extraction. Furthermore, it is obvious that not one transformation product of this type from atabrine is present, but that there must be several. The total amount of fluorescence present in the extract due to impurities appeared to be approximately 40 per cent.

If the deductions in regard to dog urine are correct, it would seem probable that the same type of transformation products is also present in human urine (patients receiving atabrine). Accordingly, extracts from the urine of three patients were washed with sodium hydroxide solution and were found to have little variation from the standard curve for atabrine.

SUMMARY

A method for the identification of certain types of organic compounds by distribution studies is proposed. The method is particularly suitable for studying biological fluids when extremely small amounts of material are present.

THE FORMATION OF PHOSPHOLIPID BY THE HEPATECTO-MIZED DOG AS MEASURED WITH RADIOACTIVE PHOSPHORUS

I. THE SITE OF FORMATION OF PLASMA PHOSPHOLIPIDS*

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Hevesy (1, 2) and Artom (3) have inferred from studies on the incorporation of labeled inorganic phosphate into phospholipid that plasma phospholipid is formed largely in the liver. This inference was based on a comparison of the specific activities of phospholipid phosphorus of plasma with those of other organs. Similar observations have been made in this laboratory (Table I). The phospholipid P^{32} per gm. of total phospholipid of several tissues of the dog was determined at 6, 18, 36, and 98 hours after the intraperitoneal injection of inorganic P^{32} (Table I). At the first interval the phospholipid P^{32} per gm. of phospholipid of the liver was higher than that of any other tissue. At the 18 hour interval the values for liver and plasma were about the same and higher than for small intestine, kidney, and muscle. At 36 hours the values for plasma and liver were still about the same, but higher than those for other tissues. At 98 hours the values for kidney and small intestine approximated those for liver and plasma.

Although the above observations do not contradict the view that the liver is the source of plasma phospholipids, interpretations based on comparisons of specific activities of phospholipid phosphorus are open to the criticism that they do not deal with a single substance but with a mixture of several different compounds. The following limitation should be noted even in the case of a single substance: the finding that the specific activity of an organ's lecithin P is lower than that of plasma serves to exclude that organ as the principal source of the plasma lecithin, but the observation that the specific activity of an organ's lecithin P is higher than that of plasma means only that the organ *may* be the source of the plasma lecithin. More definite criteria by which an organ can be determined as a source of plasma lecithin or cephalin, etc., were pointed out by Zilversmit *et al.* (4);

the reasoning applied by them for the establishment of an immediat
cursor holds equally well for the determination of an organ as source

Evidence, other than the above, cited in support of the view tha
liver is the source of plasma phospholipid is of doubtful value. It is
tionable whether the data of Nedswedsky and Alexandry support
claim that the venous blood leaving the liver has a higher phosph
content than that of the portal vein (5). Leites' data show no cons
differences in the phospholipid contents of the blood of portal vein, he
vein, and of arterial blood (6). Zilversmit et al. have shown that i
postabsorptive state 150 mg. of phospholipid are turned over per ho
the plasma of an 8 kilo dog (7). Even if liver phospholipid were the
source of the plasma phospholipid being turned over at this rate, it i
likely that measurable differences could be detected between the phos
lipid contents of the efferent and the afferent blood of the liver.

A more direct attack on the problem of the liver's rôle in the produ
of plasma phospholipid is provided by the use of the liverless animal.
the present investigation the capacity of the hepatectomized dog to syn
size phospholipid was tested with the aid of radioactive phosphorus.

Experimental Procedures

Normal Dogs (Table I)—The normal dogs were fed twice daily a mix
of lean meat, sucrose, bone ash, Cowgill's salt mixture (8), and vitamin
centrates. The feeding of this diet was begun a week before the P^{32}
injected and was not interrupted during the course of the experim
P^{32} was injected intraperitoneally as an isotonic solution of Na_2HPO_4 (
mg. of Na_2HPO_4 per cc.) containing about 0.5 to 1.0 millicurie of radi
tive phosphorus. Each dog received from 1 to 30 cc. of this solut
The dogs were sacrificed by an intracardiac injection of nembutal (30
per kilo). Blood for analysis was removed from the femoral artery
before the administration of the nembutal, while tissues were rapidly
cised immediately after its administration.

Hepatectomized and Control Dogs (Table II)—The livers of dogs w
excised by a modification of the one stage operation of Firor and Stin
(9). In the dissection of the lower part of the vena cava as well as dur
the insertion of the cannula, it was necessary to exteriorize the intestir
They were protected, however, by application of warm saline packs. 1
abdomen was not closed until the intestinal circulation was function
normally, as evidenced by the color of the intestines. In no case was t
caval or portal circulation occluded for more than 15 minutes, the usu
period being 5 to 10 minutes.

The hepatectomized dogs were kept in a warm environment. They
ceived intravenously at 30 minute intervals 5 gm. of glucose dissolved

20 cc. of isotonic saline.   Blood for analyses was removed by heart puncture.

In two experiments (Dogs FH10 and FH14) 250 cc. of cream were fed 2 hours before hepatectomy.   In all other experiments the dogs were in the postabsorptive state, the last meal having been ingested 18 hours before hepatectomy.

The *control* dogs were subjected to a sham operation; *i.e.*, manipulation of the abdominal contents without removal of the liver.   The animals were anesthetized with ethyl ether, their abdomens opened, and the visera sub-

TABLE I

Recovery of Intraperitoneally Injected P^{32} As Phospholipid P^{32} of Plasma and Tissues of Normal Dogs

Dog No.	Dog weight	Time after P^{32} injection*	Organ weight			Phospholipid content at end of experiment, mg. per 100 gm. tissue					Recovery of phospholipid P^{32}, as per cent of injected P^{32} per gm. phospholipid†				
			Liver	Kidney	Small intestine	Plasma	Liver	Kidney	Small intestine	Muscle	Plasma	Liver	Kidney	Small intestine	Muscle
	kg.	*hrs.*	*gm.*	*gm.*	*gm.*										
15	17.1	6	326	44	216	264	2730	2260	1350	850	0.13	0.37	0.25	0.14	0.0084
16	9.6	6	308	41	226	462	3000	2340	1330	1080	0.15	0.32	0.19	0.15	0.0061
14	15.0	18	284	57	198	341	2280	2100	1410	1010	0.47	0.53	0.29	0.33	
7	10.5	18	213	37	278	345	3000	2160	1070	1220	0.55	0.52	0.40	0.40	0.015
9	9.8	18	217	42	200	357	3120	2950	1040	1070	0.33	0.34	0.21	0.20	0.015
10	18.7	18	315	49	265	360	2780	2200	1160	1160	0.30	0.32	0.21	0.20	0.0092
5	9.5	36	292	52	276	219	2060	2100	1220		0.54	0.56	0.44	0.33	
6	10.2	36	246	50	266	272	2930	2620	1050	1280	0.42	0.41	0.29	0.38	0.012
17	7.0	98	228	27	201	271	2490	2430	1250	970	0.28	0.26	0.27	0.21	0.10
19	7.0	98	243	32	136	435	2580	2090	1350	1150	0.30	0.29	0.32	0.26	0.043
20	6.0	98	225	46	190	357	2740	2000	1180	1060	0.41	0.36	0.40	0.35	0.55

* Labeled Na_2HPO_4 injected intraperitoneally

† To obtain specific activities, *i.e.* $\dfrac{\text{phospholipid } P^{32}}{\text{phospholipid } P^{31}}$, multiply the values by 25.

jected to an amount of manipulation similar to that involved in hepatectomy (about 1 hour).   The abdomen was then closed.

In experiments recorded in Table II the dogs received intravenously (by femoral vein) from 2 to 10 cc. of an isotonic solution of Na_2HPO_4 (14.6 mg. of Na_2HPO_4 per cc.) containing approximately 1 millicurie of P^{32}.   The P^{32} was injected 15 to 30 minutes after the closure of the abdomen.   Blood for analyses was removed by heart puncture.

Treatment of Tissues—Blood was transferred to 15 cc. centrifuge tubes containing about 5 mg. of a heparin-urea mixture (1:19 parts).   The

blood was then centrifuged for 10 minutes in a constant speed centrifuge at 3500 R.P.M. and the plasma removed.

Uniform samples of the liver were obtained by repeated grinding of the whole liver after all the gallbladder had been excised. The kidneys minus the pelves were also repeatedly ground before samples were taken. The gastrocnemius muscles were similarly treated after removal of visible fat

TABLE II

Recovery of Intravenously Injected P^{32} As Phospholipid P^{32} of Plasma and Tissues of Hepatectomized Dog

Dog	Weight	Type of operation	Time killed after P^{32} injection	Phospholipid content at end of experiment, mg. Per 100 gm. tissue			Recovery of phospholipid P^{32}, as per cent of injected P^{32}		Kidney*	Small intestine†
							Plasma			
				Plasma	Kidney	Small intestine	Per cc., $\times 10^3$	Per gm. phospholipid, $\times 10^3$	Per gm. phospholipid	Per gm. phospholipid
	kg.		hrs.							
FH1	18.0	Hepatectomy	2	270	2380		0.1	0.3	0.25	
FH2	17.7	"	3	163	2030	1140	4	3	0.27	0.091
FH10‡	20.2	"	4	300			15	5		
˙˙14‡	20.0	"	4.5	210			19	9		
˙˙15	22.0	"	4.5	344	1820	1180	5	2	0.23	0.12
FH4	22.2	"	6	153	1470	1960	3	2	0.18	0.032
FH5	19.8	"	6	204	1870	1100	6	3	0.40	0.10
FH6	20.0	"	6	315	1860	2070	7	2	0.40	0.11
FC1	19.5	Sham operation§	6	232	2530	1120	170	72	0.43	0.12
FC2	19.5	" "	6	282	2040	1300	318	110	0.26	0.12

* Both kidneys in these dogs weighed 82 gm. (FH2), 116 gm. (FH3), 96 gm. (FH4), 82 gm. (FH5), 101 gm. (FH6), 82 gm. (FC1), 76 gm. (FC2).

† The small intestine in these dogs weighed 327 gm. (FH2), 430 gm. (FH3), 352 gm. (FH4), 360 gm. (FH5), 309 gm. (FH6), 427 gm. (FC1), 350 gm. (FC2).

‡ Fed 250 cc. of cream 2 hours before the start of the operation. All other dogs recorded in this table were in the postabsorptive state; viz., they received their last meal 18 hours before the start of the operation.

§ For a description of the sham operation see the text.

and connective tissue. The small intestine was freed of mesenteric fat and carefully washed before grinding. From 10 to 20 gm. of these tissues were usually taken for analysis.

Determination of Radiophospholipid of Plasma—5 cc. samples of plasma were added to 150 cc. of Bloor's solution (3 parts of 95 per cent ethyl alcohol to 1 part of ethyl ether) and extracted for 2 hours at 56°. The extract was decanted and the residue reextracted for 1 hour with 120 cc. (24 vol-

umes) of Bloor's solution. The mixture was then filtered. The residues and filter paper were further extracted in a Soxhlet apparatus for 8 hours with ethyl ether. The alcohol-ether and ether extracts were then combined in a Kjeldahl flask, and 1.0 cc. of a saturated Na_2HPO_4 solution as well as 50 mg. of finely ground Na_2HPO_4 was added. This was done in order to "dilute" any inorganic P^{32} which may have been carried into the extracts. The combined extracts were then concentrated under a vacuum at 56° to a volume of about 1 cc., the last 20 to 30 cc. being removed under an atmosphere of CO_2. 20 cc. of ethyl ether were now added to the Kjeldahl flask, and the mixture thoroughly agitated and transferred to a glass-stoppered Erlenmeyer flask with a small side arm. This type of flask has been described elsewhere (10). The supernatant ether layer was poured off, the lower phase being caught in the side arm. The latter was then re-extracted twice and these extracts combined in an Erlenmeyer flask that also had a side arm.

In order to remove any remaining inorganic P^{32} the volume of the combined extracts was reduced to about 15 cc. on a hot water bath (about 50°), and an excess of Na_2HPO_4 was again added, both as the saturated solution and as the powdered salt. The flask was then stoppered and the mixture violently agitated for 15 minutes; the ether phase was separated from the water phase by means of the side arm and the latter extracted twice with ethyl ether. The water phase was then tested for radioactivity. If the amount of radioactivity contained in the water phase was not reduced to negligible quantities, a second "washing" with Na_2HPO_4 was carried out. In most cases the washing procedure served only as a precautionary measure, since the previous steps were sufficient to effect a complete separation of the phospholipid P^{32} from non-phospholipid P^{32}.

The ether extract was then made up to volume and a suitable aliquot taken for precipitation of its phospholipids. The phospholipid P^{32} was measured in the manner previously described (11).

Determination of Total Phospholipid—A 5 cc. sample of plasma was treated as described above and brought to the stage where the extracts were concentrated *in vacuo* to a volume of about 1 cc. No phosphate was added before the extract was concentrated. The concentrate was then extracted with petroleum ether. Phospholipids were determined by the oxidative procedures recorded previously (12).

The procedures used for the determination of phospholipid of kidney, small intestine, and muscle have been described elsewhere (12).

Results

Recovery of Phospholipid P^{32} in Plasma, Kidney, and Small Intestine of Hepatectomized Dog (Table II)—Eight hepatectomized dogs were sacrificed

at various intervals after the intravenous injection of labeled Na_2HPO_4. The plasma, small intestine, and kidneys were then analyzed for phospholipid P^{32} and total phospholipid.

Plasma—Dogs FC1 and FC2 served as controls for the hepatectomized animals. After their abdominal cavities were opened under ether anesthesia, they were subjected to visceral manipulation after the manner described above. The P^{32} was introduced intravenously into these two dogs soon after their abdominal walls were sutured and the phospholipid P^{32} of their plasma measured 6 hours later.

The recoveries of phospholipid P^{32} are expressed as percentages of the injected P^{32} per cc. of plasma and as percentages of the injected P^{32} per gm. of plasma phospholipid. 6 hours after Dog FC1 was injected with P^{32}, the recovery of phospholipid P^{32} was 170×10^{-6} per cent per cc. of plasma or 72×10^{-3} per cent per gm. of plasma phospholipid. Higher values were found in Dog FC2; namely, 318×10^{-6} per cent per cc. of plasma and 110×10^{-3} per cent per gm. of plasma phospholipid.

The values found in the hepatectomized dogs are in marked contrast to those of the controls. As late as 6 hours after the injection, the recovery of phospholipid P^{32} was only 3 to 7×10^{-6} per cent per cc. of plasma or 2 to 3×10^{-3} per cent per gm. of plasma phospholipid (Dogs FH4, FH5, and FH6). The highest recovery of phospholipid P^{32} in the entire series of eight hepatectomized dogs was found in Dog FH14: it amounted to 19×10^{-6} per cent per cc. of plasma or 9×10^{-3} per cent per gm. of plasma phospholipid. Even this maximum recovery is but a small fraction of the recoveries observed in the control animals.

Kidney—The recoveries of radiophospholipid in the kidney were about the same in the hepatectomized and control dogs. Thus in the control dogs the phospholipid P^{32} recovered was 0.26 to 0.43 per cent per gm. of kidney phospholipid; in the hepatectomized dogs the values ranged from 0.18 to 0.40 per cent per gm. of phospholipid.

Approximately 0.5 per cent of the total P^{32} injected into the dog had been incorporated into phospholipid in both kidneys of the hepatectomized dog. Since each dog received about 4×10^8 counts per minute of P^{32}, this means that about 2 million counts per minute were recovered from both kidneys as phospholipid P^{32}. In the entire plasma of the hepatectomized animal, only about 20,000 counts per minute were recovered as phospholipid P^{32} or 5×10^{-3} per cent of the injected P^{32}. The recoveries of phospholipid P^{32} in both kidneys were about 100 times as great as in the entire plasma. These observations lead to the conclusion that kidney phospholipid is not readily available to the plasma.

Small Intestine—The recoveries of radiophospholipid in the small intestine did not differ widely in the hepatectomized and control dogs. That

there exists a barrier with respect to phospholipid between plasma and tissues such as kidney and small intestine is shown by the results obtained here.   The phospholipid P^{32} recovered in both kidneys plus small intestine amounted to approximately 1 per cent of the total P^{32} injected or about 4 million counts per minute; yet (as noted above) very little phospholipid P^{32} was recovered from the entire plasma of the hepatectomized dog.

Phospholipid Contents of Plasma and Tissues of Hepatectomized Dog—The phospholipid contents of plasma, kidney, and small intestine of the hepatectomized and control dogs are recorded in Table II.   Values for normal dogs are shown in Table I.   Significant amounts of phospholipids were present in the tissues of the dog 6 hours after excision of its liver.

DISCUSSION

The observation that radiophospholipid was recovered in the kidney and small intestine of the hepatectomized dog leaves no doubt that the liver is not the only site of phospholipid formation in the animal body.   The recoveries of phospholipid P^{32} per gm. of kidney phospholipid or per gm. of small intestine phospholipid in the liverless dog did not differ significantly from those found in the intact dog.   Yet only negligible amounts of phospholipid P^{32} were recovered in the plasma of the hepatectomized dog as late as 6 hours after excision of the liver.   These results lead to the conclusion that plasma phospholipids are derived mainly from the liver.

The finding that in the hepatectomized dog the recovery of phospholipid P^{32} per gm. of kidney phospholipid and per gm. of phospholipid in the small intestine is much higher than per gm. of plasma phospholipid is indeed striking.   6 hours after the injection of the labeled phosphate, the values for phospholipid P^{32} per gm. of tissue phospholipid were about 100 times greater in the kidney than in the plasma.   At this time the total radiophospholipid present in both kidneys and small intestine would, if delivered completely to the blood stream, raise the phospholipid P^{32} per gm. of plasma phospholipid to a value of about 0.3 per cent of the injected P^{32}.   It seems reasonable to conclude that the transfer to the plasma of phospholipids formed in the kidney and small intestine, if this occurs, is not a rapid process.   But the results do not exclude a slow transfer.   Evidence indicating that certain structures are but slowly permeable to phospholipid has been presented (13, 14).   No such barrier for the transfer of phospholipids exists, apparently, between liver and plasma.   McCarrell, Thayer, and Drinker (15) point out that the liver cells are bathed in blood plasma and in this respect are perhaps in a unique position in the mammalian body.   It is this peculiar arrangement between the liver cells and plasma that probably accounts for the early appearance of radiophospholipid in the plasma of the intact animal and the almost complete absence of radiophospholipid

in that of the hepatectomized dog. The differences in the barriers tha exist between liver and plasma on the one hand and between plasma and kidney or small intestine on the other hand are well shown in the experiment of Zilversmit *et al.* (7), in which plasma containing radiophospholipid was introduced into the blood stream of the normal dog. The uptake of radio-phospholipid by the liver greatly exceeded that by kidney and small intestine.[1]

Although it is now clearly established from studies *in vitro* that such tis-sues as liver, kidney, and brain can form both ester bonds of phosphate in the phospholipid molecule (10, 16, 17), the recovery of radiophospholipid in a given tissue of the hepatectomized or intact dog provides no informa-tion on the exact reaction carried out by the given tissue. The radiophos-pholipid or newly formed phospholipid in the kidney and small intestine of the hepatectomized dog is probably not derived from plasma, for the specific activities of the phospholipid found in the plasma of this preparation were much lower than those in the kidney and small intestine. Since in the hepatectomized dog practically no radiophospholipids were delivered to these tissues by the plasma, the recovery of phospholipid P^{32} in the kidney and small intestine does demonstrate that at least the last step in the forma-tion of the phospholipid molecule can occur in each of these tissues *in vivo*. This last step need not involve the formation of the ester bonds of phosphate. For example, it is conceivable that the conversion of inorganic P^{32} to glycerophosphorylcholine P^{32} takes place in one tissue and that this compound is then carried to another tissue where by the addition of fatty acids it is converted to phospholipid. No evidence in support of such mechanism, however, exists at the present time.

SUMMARY

The rôle of the liver in the formation of plasma phospholipids was inves tigated. Inorganic P^{32} was injected into normal and hepatectomized dog and its recovery as phospholipid P^{32} compared in these animals.

1. Plasma phospholipids are formed mainly in the liver. Excision of the liver reduced the recovery of phospholipid P^{32} of the plasma to very small quantities.

2. The recovery of phospholipid P^{32} in kidney and small intestine was not reduced by excision of the liver. Apparently phospholipids synthesized by these two tissues are not readily available to the plasma.

[1] At the end of 5 hours, the liver contained approximately 1500 counts per minute per gm.; kidney and small intestine contained 600 and 300 counts per minute per gm., respectively.

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FERRITIN

V. X-RAY DIFFRACTION DATA ON FERRITIN AND APOFERRITIN

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Ferritin and apoferritin (1–3) from horse spleen were made available to the writer by Dr. Michaelis and Dr. Granick of The Rockefeller Institute for Medical Research. Both crystallize in isotropic octahedra. The individual crystals were too small for single crystal x-ray studies but because of the cubic character of the crystals, it was possible to make use of the powder method. The Debye-Scherrer patterns of ferritin and apoferritin appear at first sight very different. However, this difference (Table I) is only one of line intensity. The locations of the lines, both for ferritin and apoferritin, correspond to those one would expect from a face-centered cubic cell, side 186 Å. Many lines are, of course, missing, but enough are found to confirm this choice of unit cell for both proteins. Apoferritin is obtained (3) by removing the iron completely from ferritin. The iron forms 23 per cent of the dry weight of ferritin. It is a remarkable fact that the removal of this large amount of iron causes no observable change in the unit cell size.

Apoferritin appears to be homogeneous in the ultracentrifuge[1] (in contrast to ferritin), with a molecular weight of about 500,000. The density of wet ferritin is about 1.45 and of wet apoferritin 1.27 as measured by flotation methods. Wet crystals were measured on a microscope stage and allowed to dry in air. The linear shrinkage both for ferritin and apoferritin was 12.5 per cent. This corresponds to a decrease by volume of 33 per cent. The volume of the unit cell is (186 Å.)^3; *i.e.,* 6,435,000 cu. Å. For a density of 1.27, the cell weight is $6,435,000 \times 1.27 \times 10^{-24}$ gm. $= 8,180,000 \times 10^{-24}$ gm. If one assumes that the decrease in volume upon drying is all due to the loss of water, then the mass of the water lost upon drying is $6,435,000 \times 0.33 = 2,130,000 \times 10^{-24}$ gm. The anhydrous weight of the unit cell is then $6,050,000 \times 10^{-24}$, or, in units of molecular weight, 3,670,000. Assuming that the molecular weight, 500,000, as determined by the ultracentrifuge is approximately correct, then there are 8 molecules per cell and the anhydrous x-ray molecular weight is 460,000. This is an upper limit, as no allowance was made in the above computation for the presence of salt or for water in interstices.

[1] Rothen, A., paper in preparation.

A similar computation can be made for the ferritin. The density is about 1.45. This gives an anhydrous molecular weight of 545,000. The corresponding "wet" molecular weights are 620,000 for apoferritin and 706,000 for ferritin.

These results agree fairly well with those obtained by other methods. The value 460,000 for anhydrous apoferritin agrees with the value 500,000 obtained from ultracentrifuge data. The ratio of the molecular weight of anhydrous apoferritin to ferritin is 84 per cent, while from the analytical data of Michaelis and Granick it should be 77 per cent. The possibility of serious errors in the above computations is admittedly great. It has been shown (4) recently that densities of protein crystals measured by flotation methods (the method used for this work) are subject to consid-

TABLE I

X-ray Diffraction Data on Ferritin and Apoferritin

hkl	$d_{comp.}$ $a = 186$ Å.	Ferritin		Apoferritin	
		$d_{obs.}$	Intensity	$d_{obs.}$	Intensity
111	107.5	109	v.v.s.	110	w.-m.
200	93	93	w.	92	w.
220	65.8	66.0	v.w.	66.0	s.
311	56.2			56.5	m.
222	53.8				
400	46.5				
331	42.8			42	w.
420	41.7				
422	38.0				
511, 333	35.8	35.9	v.v.w.		

erable error. It is even possible that, because much too low an estimate of water content may have been made, there are only 4 and not 8 molecules per unit cell. Lacking single crystals large enough to make reliable hydration measurements upon (4), one must use less direct methods of estimating the hydration. The method of linear shrinkage here used certainly gives a minimum value for the hydration and consequently a maximum for the anhydrous molecular weight of the unit cell.

The fact that ferritin and apoferritin both crystallize in cubic cells of the same size suggests strongly that we are dealing with identical (as far as size and shape are concerned) protein molecules. The iron content of ferritin is then contained in the large interstices between molecules. If the iron were specifically attached to active points on the protein molecules, one would not expect unit cells of identical size for ferritin and apoferritin. The marked differences in intensity in the x-ray diagrams are most likely

due to the iron content of ferritin.   It has not yet been possible to base any conclusions as to the locations of the iron-filled lacunae upon these intensity differences.   Should crystals large enough for single crystal x-ray studies become available, it may then be possible to say something concerning the arrangement of the protein molecules.

SUMMARY

X-ray powder diagrams of ferritin and apoferritin show that both materials crystallize in face-centered cubic structures of identical cell size, differing only in line intensity.   This suggests that the protein is the same in both substances and that it is the packing of these identical molecules which determines the cell size, a packing which is not disturbed by the presence of the iron.

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A RAPID COLORIMETRIC METHOD FOR THE DETERMINATION OF GLYCOLS IN AIR*

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The utilization of propylene glycol (1) and triethylene glycol (2,2'-ethylenedioxydiethanol) (2) for sterilization of air in occupied rooms has made necessary the development of a simple, rapid method for the determination of these substances in the atmosphere. The analytical methods available for propylene glycol (3, 4) involve time-consuming procedures. In the present method glycol contained in the air is absorbed in water and analyzed in solution by quantitative oxidation with acidified potassium dichromate. The green color of the chromic ion in the resulting solution, the intensity of which increases with the amount of reduced potassium dichromate, is then used as an index of the concentration of glycol originally present. The analysis is simple, rapid, and, therefore, especially suitable for field studies. The method should be applicable also to the determination of other organic compounds which are quantitatively oxidized by potassium dichromate in acid solution.

Air-Sampling Method—Air containing glycol is drawn through two Folin aeration tubes (5)[1] connected in series. Each aeration tube is inserted through a 2-hole rubber stopper into a test-tube (outside diameter 32 mm., length 200 mm.) with the bulb placed close to the bottom. A fine scratch is made on the test-tube at a point corresponding to 15 ml. of liquid and approximately 12 ml. of water added. The aeration tubes are connected in series to a pump[2] (Fig. 1). The volume of air passing through the water in the absorption tubes is measured with a flowmeter, such as that shown in Fig. 1 which has been found especially convenient for this purpose.[3] The air sample taken must be large enough to furnish sufficient glycol for accurate analysis. Because of the relatively low concentrations generally employed for air sterilization with triethylene glycol, 300 liters of air are required when this compound is used; for propylene glycol, a 50 liter

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[1] Available at commercial supply houses.

[2] Silent, efficient pumps are purchasable from the W. M. Welch Manufacturing Company, Chicago.

[3] Lemon, H. M., and Wise, H., to be published.

sample of air is adequate.   Air may be passed through the absorption apparatus at a rate of 20 to 30 liters per minute.   A higher rate of sampling will cause excessive splashing of the liquid.

At the end of the sampling, the aeration tubes are disconnected and any glycol which may have been deposited in them is washed down into the test-tubes.   This may be accomplished by drawing the liquid up into the aeration tube several times.   The Folin tubes are then lifted out of the solution, rinsed with 1.5 to 2 ml. of distilled water, and the washings collected in the respective absorption tubes.   The inside of the flowmeter mentioned above (Fig. 1) must also be rinsed in this process.   Finally, enough water is added to each test-tube to bring the liquid level to the 15 ml. mark and, after mixing, aliquots are withdrawn for analysis.   For higher
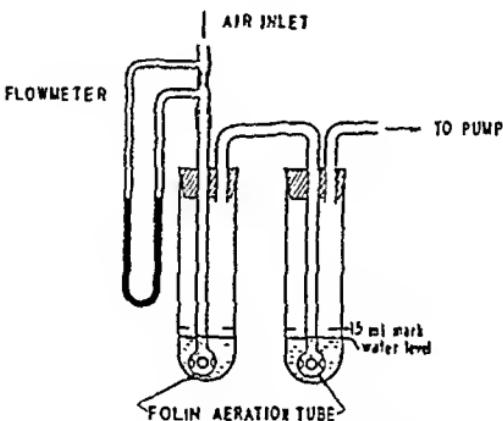
FIG. 1. Air-sampling arrangement for analysis of glycol vapors

accuracy we have adopted the practice of analyzing the contents of each tube separately.

Analytical Procedure

Reagents—
Potassium dichromate, analytical reagent.
Sulfuric acid, analytical reagent, specific gravity 1.84.

2.00 gm. of potassium dichromate are dissolved in 20 ml. of distilled water and the solution is made up to 1000 ml. with concentrated sulfuric acid. The concentration of potassium dichromate in this solution will be such that 10 ml. will oxidize a maximum of about 2.4 mg. of glycol.   The solution should be kept in a glass-stoppered bottle in the dark.

Glycol Standards—1.0 gm. of the test glycol is diluted to 1000 ml. with distilled water, so that 1 ml. of the resulting solution is equivalent to 1 mg. of glycol.   For field work it has been found satisfactory to use the "regular"

grade of triethylene glycol and the "special" grade of propylene glycol[4] which are sufficiently pure for this determination.

Procedure—Aliquots are removed from each of the two absorption tubes and pipetted into Pyrex test-tubes (outside diameter 25 mm., length 200 mm.). In order for oxidation to be complete the aliquot should not contain more than 2.4 mg. of either glycol. 10 ml. of solution are satisfactory for the analysis of triethylene glycol but in the test for propylene glycol 2 ml. are taken and diluted to 10 ml. because of the much higher concentrations in which this latter substance is generally employed in the air. The test-tubes are placed in a wire basket which is kept in a cold water bath while 10 ml. of the potassium dichromate-sulfuric acid reagent are added slowly to each sample. After thorough mixing of the solution the tubes are heated in
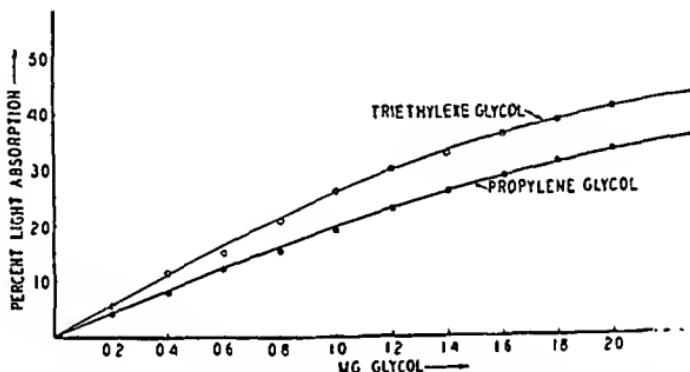
FIG. 2. Calibration curves for colorimetric analysis for propylene glycol and triethylene glycol.

a boiling water bath for 15 minutes. In order to minimize evaporation losses they are covered with small watch-glasses or with glass bulbs which fit loosely on top of the test-tubes. At the end of the heating period the tubes are cooled to room temperature by immersing the wire basket in a cold water bath. The color of the samples is now compared with that of known standards, either with a photoelectric colorimeter or by visual means.

For the preparation of color standards a blank and twelve glycol samples ranging from 0.2 to 2.4 mg., in steps of 0.2 mg. of glycol, are measured into Pyrex test-tubes (outside diameter 25 mm., length 200 mm.) and diluted with distilled water to give a total volume of 10 ml. To each of these solutions 10 ml. of potassium dichromate-sulfuric acid reagent are added and the same analytical procedure carried out as described above.

The color comparison can be made most accurately with a photoelectri

[4] As supplied by the Carbide and Carbon Chemicals Corporation, New York.

colorimeter.[5] The intensity of the green color due to the presence of
chromic ions is determined by means of a No. 620 Evelyn glass filter,
which has a maximum transmission at a wave-length of 616 mμ. A series
of determinations with the known standards is run, and from these data
calibration curves for propylene glycol and triethylene glycol, respectively,
are constructed (Fig. 2). The per cent light absorption of the solution
with unknown glycol concentration is then read off from these curves
directly in terms of mg. of glycol. It will be noted that the calibration
curves here presented do not obey Beer's law; however, they are repro-
ducible and average deviations from the mean do not exceed ±3 per cent.

If a photoelectric colorimeter is not available, satisfactory results may be
obtained by visual color comparison of the unknown solutions with the
glycol standards. 9 ml. of the cooled solutions, standards and unknowns
respectively, are pipetted into 10 ml. test-tubes (outside diameter 13 mm.,
length 100 mm.) and the color of the unknown matched against that of the
standards by looking down into the open end of the tubes held side by side
above a white background.   In this way a liquid column 90 mm. long is used
for the comparison, so that a fairly high degree of accuracy may be achieved.
Unscratched test-tubes of uniform bore and thickness should be used for this
determination.

Very good agreement has been found between the visual and photoelectric
methods of color comparison.   Thus, with air samples of the magnitude here
recommended the solution analyzed will contain between 1.0 and 2.0 mg. of
propylene or triethylene glycol. It is possible to match the color visually
with an accuracy of ±0.10 mg. With a photoelectric colorimeter an ac-
curacy of ±0.05 mg. may be achieved.

DISCUSSION

Oxidation with acidified potassium dichromate is a standard method for
the determination of glycerol (6) and has been used for other organic hy-
droxy compounds. In such determinations it has been the practice to
measure the disappearance of the dichromate ion, either colorimetrically
(6, 7) or by means of a back titration (8, 9). When small quantities of oxi-
dizable material are being determined, a large excess of potassium dichro-
mate remains in the solution. To measure accurately the change in absorp-
tion due to the dichromate ion (wave-length region of 420 to 460 mμ)
under these conditions, it becomes necessary to dilute the solution greatly
in order to obtain sufficient light transmission so that the region of max-
imum sensitivity of the photoelectric system is being utilized.   This pro-

[5] In this laboratory we have used the Evelyn colorimeter, Rubicon Company,
Philadelphia.

cedure can be avoided without any significant loss of sensitivity by using the appearance of the chromic ion as a measure of the extent of oxidation which has occurred, rather than the disappearance of the dichromate ion.

Because of the non-specificity of the oxidation reaction care must be taken to insure the absence of other reducing substances which will react with the potassium dichromate-sulfuric acid solution. Air samples taken in various rooms free from glycol produce some reaction when treated with

TABLE I

Absorption of Triethylene Glycol in Air by a Single Folin Aeration Tube and by Two Tubes Connected in Series

Each tube contains 15 ml. of distilled water. All air samples are 300 liters.

Experiment No.	Rate of air passage	Total concentration of glycol found	Per cent absorbed in 1st Folin aeration tube	Per cent absorbed in 2 Folin aeration tubes in series
	l. per sec.	*mg. per l.*		
1	0.33	0.023	83	97
2	0.33	0.023	80	97
3	0.40	0.019	77	96
4	0.37	0.021	68	92
5	0.33	0.008	66	90
6	0.50	0.007	69	92
7	0.53	0.011	76	95
8	0.33	0.008	65	88
9	0.33	0.012	61	86
10	0.49	0.007	64	87
11	0.34	0.010	67	91
12	0.33	0.010	66	90
13	0.34	0.008	70	91
14	0.34	0.010	66	86
15	0.34	0.010	65	88
16	0.34	0.018	64	92
17	0.34	0.015	70	94
Average			69 ± 5	91 ± 3

this oxidizing agent. The values of the blanks so obtained will vary for different locations, depending upon the amount of dust, sulfur dioxide, and other reducing substances present in the air. Generally, however, we have found that these blanks do not exceed amounts corresponding to 0.0006 mg. per liter of glycol for air of occupied rooms and 0.0003 mg. per liter of glycol for outside air. This quantity is completely negligible in work with propylene glycol, but in atmospheres containing much foreign matter it may achieve significant proportions when triethylene glycol is tested for. It is advisable, therefore, to determine the blank values before glycol is

introduced into the rooms to be studied, or to take air samples under identical conditions in control rooms, to compensate for the error caused by reducing substances other than glycols. Especially high blanks are found occasionally in air from the laboratory, which may contain large amounts of reducing materials such as ethyl alcohol, acetone, formaldehyde, etc.

Tests have shown that when air containing triethylene glycol is dispersed through 15 ml. of water by means of a single Folin aeration tube at a rate of 0.33 liter per second or higher only 69 ± 5 per cent of the total glycol content of the air is absorbed in the solution (Table I). However, when two absorption tubes are connected in series, each filled with 15 ml. of distilled water and with the same rate of air passage, the per cent absorption in the two combined tubes is increased to 91 ± 3 per cent (Table I). Although a small amount of triethylene glycol still remains unabsorbed after passage through two absorption tubes (as may be demonstrated by the use of three tubes in series), the order of magnitude of this error approaches the accuracy of the analytical method, and so the additional inconvenience involved in the use of three tubes does not seem to be warranted.

Sampling by means of a single aeration tube with the addition of a constant correction factor of 31 per cent to allow for incomplete absorption does not seem advisable, because of the greater variability in the percentage retained in one tube over that absorbed in two tubes, as shown in Table I. Such a procedure could be used, however, if only an approximate value of the amount of triethylene glycol vapor in air were desired. The use of two tubes also tends to compensate for variations due to differences in the structure of individual aeration tubes.

Tests with propylene glycol reveal that the absorption of this substance in water by two Folin aeration tubes is more complete than with triethylene glycol. After passage through two absorption tubes only a small amount of propylene glycol remains unabsorbed and this quantity is well within the experimental error of the method, so that no correction factor is necessary.

SUMMARY

1. A sampling method for glycol contained in air is described. It consists of passing the test air at a rate of 0.3 to 0.5 liter per second through two Folin aeration tubes connected in series. A volume of 50 liters of air is taken for analysis for propylene glycol and 300 liters for triethylene glycol because of the difference in the concentration of these two substances generally used in air sterilization.

2. A colorimetric method for the determination of triethylene and propylene glycols is presented involving oxidation by acidified potassium dichromate and measurement of the green color of the resulting chromic ion. The reaction is non-specific; therefore a blank determination is necessary.

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AN ETHER EXTRACTION METHOD FOR THE
DETERMINATION OF BLOOD PHENOLS

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Experiments previously reported from this laboratory (1) have shown that less than 1 per cent of the total "diazo value" of blood is actually made up of ether-soluble phenolic compounds. In the present work, an attempt was made to develop a more satisfactory method for the determination of the blood phenols. The apparatus and analytical methods are, in general, similar to those previously described for the determination of urine phenols (2).

Two difficulties are inherent in any quantitative study of blood phenols; namely, the very small concentration of true phenols in the blood and the difficulty of removing the blood proteins without loss of phenolic compounds. According to Voinar and Babkin (3) phenol added to serum could not be detected in the corresponding filtrates unless added in a quantity above 1 mg. per cent, owing to adsorption by the protein precipitate. However Deichmann and Schafer (4) report that added phenol can be quantitatively recovered in tungstic acid blood filtrates.

EXPERIMENTAL

Removal of Blood Proteins and Recovery of Added Phenolic Compounds—
12 cc. portions of pooled, oxalated blood obtained from the hospital laboratory[1] were pipetted into 100 cc. centrifuge tubes. Measured quantities of an aqueous phenol solution ranging from 0.003 to 0.4 mg. of phenol (equivalent to 0.025 to 3.333 mg. per cent) were added to the blood samples. The tubes were then shaken gently for several minutes. Sufficient distilled water was added to bring the volume of added water up to 24 cc. The blood was then deproteinized, and the supernatant fluid analyzed for free phenol content by the procedure outlined later. The precipitates were stirred with about 35 cc. of distilled water for several minutes and then recentrifuged. The supernatant fluids were decanted into extraction tubes, 1 cc. portions of 10 N sulfuric acid added, and then ether extraction and phenol analysis effected in the usual manner.

The data in Table I show that the protein-free, supernatant fluids con-

[1] The author is indebted to Miss M. Johnson, Miss D. Stern, and Mr. A. Szczpinski of the University Hospital Laboratory Staff for their helpful cooperation.

TABLE I

Recovery of Phenol Added to Blood

Experiment No.		Extraction of blood filtrates					Extraction of protein-tungstate ppts.				Per cent total recovery
	Dilution	Free phenols in control	Phenol added	Phenol found	Phenol recovered	Per cent recovery	Free phenols in control	Phenol found	Phenol recovered	Per cent recovery	
		mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*		
1	1:5	0.024	3.333	2.650	2.626	78.8	0.0034	0.600	0.5966	17.9	96.7
2	1:10	0.024	3.333	2.700	2.676	80.3	0.0034	0.530	0.5266	15.8	96.1
3	1:5	0.024	1.667	1.304	1.280	76.8	0.0034	0.294	0.2906	17.4	94.2
4	1:10	0.024	1.667	1.370	1.346	80.7	0.0034	0.232	0.2286	13.7	94.4
5	1:5	0.026	0.417	0.330	0.304	72.9	0.0020	0.085	0.0830	19.9	92.8
6	1:10	0.026	0.417	0.337	0.311	74.6	0.0017	0.064	0.0623	14.9	89.5
7	1:5	0.030	0.167	0.155	0.125	74.9	0.0030	0.042	0.0390	23.3	98.2
8	1:10	0.025	0.167	0.156	0.131	78.4	0.0030	0.034	0.0310	18.6	97.0
9	1:5	0.020	0.167	0.147	0.127	76.0	0.0027	0.033	0.0303	18.1	94.1
10	1:10	0.041	0.167	0.169	0.128	76.6	0.0033	0.036	0.0327	19.6	96.2
11	1:5	0.012	0.080	0.073	0.061	76.3	0.0043	0.017	0.0127	15.9	92.2
12	1:5	0.011	0.080	0.070	0.059	73.7	0.0043	0.017	0.0127	15.9	89.6
13	1:5	0.014	0.042	0.049	0.030	71.4	0.0024	0.008	0.0024	13.3	84.7
14	1:5	0.013	0.025	0.033	0.020	80.0	0.0024	0.005	0.0026	10.4	90.4

TABLE II

Recovery of p-Hydroxybenzoic Acid Added to Blood

Experiment No.	Extraction of blood filtrates					Extraction of protein-tungstate ppts.				Per cent total recovery
	Free p-hydroxybenzoic acid in control blood	p-Hydroxybenzoic acid added	p-Hydroxybenzoic acid found	p-Hydroxybenzoic acid recovered	Per cent recovery	Free p-hydroxybenzoic acid in control	p-Hydroxybenzoic acid found	p-Hydroxybenzoic acid recovered	Per cent recovery	
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*		*mg. per cent*	*mg. per cent*	*mg. per cent*		
1	0.040	1.667	1.231	1.191	71.4	0.0170	0.333	0.316	19.0	90.4
2	0.040	0.834	0.660	0.620	74.3	0.0083	0.200	0.1917	23.0	97.3
3	0.030	0.417	0.350	0.320	76.7	0.0200	0.083	0.063	15.1	91.8
4	0.030	0.417	0.340	0.310	74.4	0.0083	0.0757	0.0674	16.2	90.6
5	0.028	0.167	0.166	0.138	82.6	0.0083	0.0410	0.0327	19.6	102.2
6	0.025	0.084	0.087	0.062	73.8	0.0093	0.0278	0.0185	22.0	95.8
7	0.022	0.084	0.087	0.065	77.4	0.0080	0.0300	0.0220	26.2	103.6
8	0.038	0.042	0.070	0.032	76.2	0.0108	0.0167	0.0059	14.0	90.2
9	0.034	0.042	0.065	0.031	73.8	0.0125	0.0250	0.0125	29.7	103.5

tain 71.4 to 80.7, average 76.5 per cent, of the added phenol, the remainder, 10.4 to 23.3, average 16.76 per cent, being retained by the coagulum. Thus a total of 84.7 to 98.2, average 93.3 per cent, of the added phenol is accounted

for in the two determinations. The data also show that a 1:5 dilution of the blood gives the same results as a 1:10 dilution; however, the more concentrated filtrate makes for more satisfactory hydrolysis and extraction.

Similar experiments were conducted with p-hydroxybenzoic acid added to blood. The data in Table II show that 71.4 to 82.6, average 75.6 per cent, of the acid is found in the supernatant fluid, the balance in the coagulum. In general, similar results were obtained with added p-cresol and p-hydroxyphenylacetic acid; however, the values obtained were somewhat more variable owing, in part, to difficulties in color matching. Since the data show that adsorption is fairly independent of the concentration of added phenol and p-hydroxybenzoic acid, a correction factor can be used, thus eliminating the necessity of routinely analyzing the coagulum. The correction factor for blood phenols was taken as 0.76.

Procedures for Analysis of Blood Phenols

Procedure for Free Phenols—12 cc. of oxalated blood are diluted with 24 cc. of distilled water in a 100 cc. centrifuge tube. After several minutes, 12 cc. of 10 per cent sodium tungstate are added and then, gradually, 12 cc. of $\frac{2}{3}$ N sulfuric acid. The contents are stirred occasionally for about 15 minutes and then rapidly centrifuged for another 15 minutes. Sometimes it is necessary to decant the supernatant filtrate into a clean tube and recentrifuge in order to remove the last traces of precipitate. Filtration cannot be used because even ether-extracted filter paper contains appreciable quantities of compounds which react with the diazo reagents. In addition, centrifugation results in a much greater yield of filtrate, thus conserving the amount of blood required for the determination.

An accurately measured quantity of filtrate (36 to 40 cc.) is transferred to the coaxial extraction tube (1, 2), followed by 1 cc. of 10 N sulfuric acid. The pH of the solution is approximately 1. Freshly distilled ether (about 6 to 9 cc.) is added until the ether layer just overflows the inner return tube.[2] The extraction tube is now attached to the condenser. A few particles of pulverized porcelain (to prevent bumping) and about 3 cc. of ether are added to a small receiver, which is then attached to the extraction tube and partially immersed in water maintained at 70–75°. The solid glass stopper is placed *loosely* in the top of the condenser. Apparatus with glass joints is required because cork or rubber yields diazo-chromogenic materials.

[2] Blood filtrates contain relatively large quantities of water-soluble compounds, especially histidine and tyrosine, which react with the diazo reagents; hence particular care must be exercised at all times that none of the fluid being extracted is spilled down the inner return tube. Although aqueous solutions of histidine and tyrosine at various pH values were extracted with ether for several hours, the extracts were always found to be negative if contamination was avoided.

A fairly rapid, continuous extraction is maintained for 2 hours. The water bath is removed, and the apparatus shaken gently until sufficient ether overflows the inner return tube to bring the contents of the receiver up to the 3 cc. mark. The ether extract is diluted with 5 cc. of 95 per cent alcohol. 5 cc. portions of several alcoholic phenol standards (0.001 to 0.005 mg. of phenol per 5 cc.) are transferred to test-tubes, each containing 3 cc. of ether. 2 cc. of water, 1 cc. of diazotized p-nitroaniline,[3] and 3 cc. of 5 per cent sodium carbonate are added. The solutions are mixed and then matched in the colorimeter. Blood particularly rich in free phenolic compounds may require 6 or even 9 cc. of ether for the extraction. The extract is then diluted with 10 or 15 cc. of 95 per cent alcohol and an 8 cc. aliquot analyzed as above. In each case the value obtained is divided by the factor 0.76 to correct for the phenolic compounds adsorbed by the coagu-

TABLE III

Determination of Phenolic Content and "Diazo Value" of Mixed Human Blood

Experi- ment No.	Free phenols (a)	Conjugated phenols (b)	Total phenols	Total phenols (a) + (b)	Free "diazo value"		Total "diazo value"	
					As histidine	As phenol	As histidine	As phenol
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*	*mg. per cent*
1	0.0421	0.0659	0.1040	0.1080	6.3	1.2	7.9	1.9
2	0.0184	0.0448	0.0630	0.0632	4.2	1.1	4.5	1.4
3	0.0243	0.0553	0.0750	0.0796	5.0	1.4	6.3	1.8
4	0.0184	0.0400	0.0553	0.0584	5.0	1.2	6.3	1.5
5	0.0400	0.0695	0.1200	0.1095	6.0	1.4	7.4	1.7
6	0.0630	0.0659	0.0990	0.1289	6.9	1.6	7.8	1.9

lum. Since the reaction shows poor proportionality at these low concentrations, standard and unknown should have approximately the same concentration.

Procedure for Conjugated Phenols—Most of the ether layer is removed with a pipette and discarded. The residue in the extractor is then decanted into a 100 cc. round bottom flask which has a 24/40 joint. A few beads are added and the ether volatilized from a hot water bath. If the ether is allowed to remain for several hours before volatilization, chromogenic substances which react with the diazo reagents may be formed. The flask is immediately attached to a condenser and gently refluxed for 1 hour. The cooled hydrolysate still has a pH of approximately 1. It is then decanted

[3] 25 cc. of the p-nitroaniline solution (1.5 gm. of the crystalline material in 500 cc. of water containing 40 cc. of concentrated HCl) are added to 1.5 cc. of a 5 per cent sodium nitrite solution. This reagent will remain clear and reactive for several days if kept in the cold.

back into the extraction tube and again extracted with 3 cc. of ether for 2 hours. The ether extract is then analyzed for its phenolic content in the usual manner.

Procedure for Total Phenols—If sufficient filtrate is available, the total phenols also can be determined. 40 cc. of filtrate are hydrolyzed for 1 hour with 1 cc. of 10 N sulfuric acid. The hydrolysate is then extracted with ether for 2 hours in the usual manner.

Phenolic Content and "Diazo Value" of Blood—In these experiments several bloods, which were essentially normal clinically, were pooled and subjected to analysis as outlined above. In addition the "diazo values" by the Theis-Benedict (5) method, as described previously (1), were determined. The data in Table III show that the free phenols range from 0.0184 to 0.063 mg. per cent, the conjugated phenols from 0.040 to 0.0695 mg. per cent, and the total phenols from 0.055 to 0.12 mg. per cent. The values obtained for the total phenols check fairly well with the sum of the free and conjugated phenols. The various "diazo values" obtained are similar to those reported in previous work (1).

Separation of the ether-soluble compounds into phenols and aromatic hydroxy acids, as carried out with urine phenols (2), was not found to be practical, owing to their low concentration in the blood. For the same reason great accuracy cannot be claimed for the present method. It is believed, however, that the method does yield essentially correct values.

SUMMARY

An analytical method for blood phenols is described in which these bodies are extracted from the blood filtrate with ether and then determined directly in the ether extract with diazotized *p*-nitroaniline. A correction factor is necessary, since about 25 per cent of the various phenols and aromatic hydroxy acids is adsorbed during deproteinization. Values are given for free, conjugated, and total blood phenols.

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THE RÔLE OF RIBOFLAVIN IN BLOOD REGENERATION*

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Several reports in the literature suggest a possible relationship between riboflavin deficiency and anemia. Intramuscular administration of purified flavin prepared from liver was reported by Stare and Thompson (1) to have no effect on hemoglobin, erythrocyte, or reticulocyte response in patients with pernicious anemia. However, Miller and Rhoads (2) were able to produce in dogs a syndrome similar to sprue by diets deficient in riboflavin. These investigators (3) later reported the finding of an antipernicious anemia principle in egg white and in a rice polishings concentrate low in riboflavin, which confirmed the view of other workers that riboflavin is not the extrinsic factor of the deficiency state in pernicious anemia. Kohls (4) found evidence in blood studies for the association of anemia with a deficiency of the vitamin G complex (presumably a riboflavin plus). A slight anemia was listed by Potter *et al.* (5) as one of the symptoms of this deficiency in dogs. György and coworkers (6) found that riboflavin causes a definite increase in hemoglobin production above the basal level when fed to standardized anemic dogs on a salmon-bread diet.

Studies in this laboratory (7) on hemoglobin regeneration in dogs with a synthetic ration free of the B complex vitamins, supplemented only with the crystalline B vitamins, have given ample proof of the adequacy of this ration for good hemoglobin production during long periods of phlebotomy. Such a ration, then, makes possible the investigation of the rôle of the B complex vitamins in blood regeneration, because it allows the use of a ration relatively free of any one member.

Methods and Materials

Seven mongrel dogs of heavy breed were used in this experiment. Four of these were recently weaned litter mate puppies (Dogs 251 to 254) approximately 8 weeks of age. Two dogs (Nos. 236 and 238), litter mates about 4 months of age, had previously been on a choline-deficient ration but had shown no outward signs of any deficiency. Dog 186 was an adult

dog which had been on the synthetic ration for some time. The puppies were twice treated with an anthelmintic (tetrachloroethylene) before being placed on the synthetic ration. The synthetic ration had the following composition: sucrose 66, casein (acid-washed) 19, cottonseed oil 8, cod liver oil 3, Salts 4, 4 per cent. Salts 4 is the same as Salts 1 of Phillips and Hart (8) with an additional 1.21 gm. of $MnSO_4 \cdot 4H_2O$ per kilo of salt mixture. The ration was supplied *ad libitum* and daily food consumption records were kept. This ration was supplemented with the following crystalline vitamins: thiamine 100 γ, pyridoxine 60 γ, calcium pantothenate 500 γ, niacin 2 mg., and choline 50 mg. per kilo of body weight per day. These vitamins in aqueous solution were fed twice weekly by pipette. Body weights were taken regularly before the morning feeding.

Blood samples for analysis were always taken from the radial vein. A 20 cc. sample was drawn into a syringe, the dead space of which was filled with Wintrobe's non-shrinking oxalate. Hemoglobin determinations were carried out in duplicate by the method of Evelyn (9). In addition, routine red blood cell counts were made and hematocrit values were obtained with Wintrobe tubes. During the periods of phlebotomy blood was removed at regular weekly intervals from an external jugular vein. Whenever possible, 25 per cent of the estimated total blood volume was removed at each bleeding. The volume removed was then measured and the hemoglobin determined. The total blood volume was considered to be 8 per cent of the body weight and the total hemoglobin was calculated from the hemoglobin level of the blood. These values, together with the known amount of hemoglobin removed in the analysis sample and during the periods of phlebotomy, permitted the calculation of the total "hemoglobin made."

<div style="text-align:center">EXPERIMENTAL</div>

Figs. 1, 2, and 3 give the complete data for the individual dogs on body weight, food consumption, hemoglobin per cent, total hemoglobin, and hemoglobin removed. Fig. 1 (Dog 251) is representative of the data obtained on the young growing dogs. Fig. 2 shows the response of Dog 236 and includes the results obtained with liver feeding. Fig. 3 (Dog 186) shows the typical results obtained with an adult dog. Fig. 4 summarizes the data on the amount of bleeding and the hemoglobin production according to periods of different levels of riboflavin feeding.

After 9 to 12 weeks on the synthetic ration without riboflavin four of the dogs (Nos. 251, 252, 254, and 238) developed a severe anemia. The hemoglobin was reduced to about 6 gm. per 100 cc. of blood. In the same length of time the remaining three dogs showed a milder anemia, with the hemoglobin reduced to about 9 gm. per 100 cc. of blood. In all cases the anemia was hastened by phlebotomy, as indicated in Figs. 1, 2, and 3.

When, at the end of 9 weeks, a hemoglobin level of 5.9 gm. per 100 cc. was reached in Dog. 251, phlebotomy was restricted to analysis samples of 20 cc. volume. The anemia became stabilized at a level of 6.8 gm. per 100 cc. 5 γ of crystalline riboflavin per kilo of body weight per day were then administered orally. This low level of riboflavin fed over a week was obviously insufficient. There was a further reduction in the hemoglobin level and a loss of body weight. The response to riboflavin levels of 10 and 15 γ was also negligible. The severity of the riboflavin deficiency
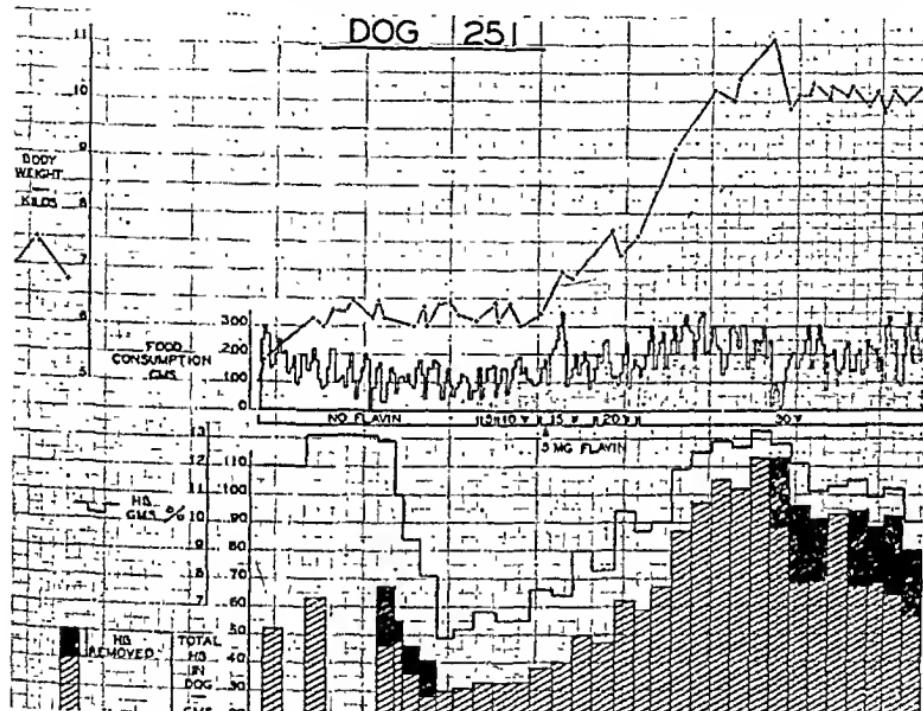
FIG. 1. Experimental history of a typical young growing dog (No. 251).. The supplements of riboflavin are given as γ per kilo of body weight per day.

became obvious by this time and the dog was found prostrate and suffering from spasmodic muscular convulsions. An intravenous injection of 5 mg. of riboflavin caused a rapid recovery. The response in hemoglobin and weight and the increased food consumption during the remainder of the period of feeding 15 γ were probably due to this injection. The slow and erratic rise in hemoglobin continued during the feeding at the 20 γ level. It was not until an increase to 30 γ of riboflavin was made that the dog responded positively and continuously in hemoglobin production. In 3 weeks the hemoglobin rose from 9.6 to 12.5 per cent; a total of 45.5 gm. of hemoglobin was made. During 4 additional weeks the maximum hemo-

globin of 13.3 per cent, probably close to the normal for the animal, was reached.

This dog was then continued on 30 γ per kilo daily, having attained a stable normal hemoglobin level, and was bled at regular weekly intervals to the extent of about 25 per cent of the blood volume. During a period of 8 weeks of phlebotomy a total of 176 gm. of hemoglobin was removed (actual determination) and 133.3 gm. were made (as calculated).
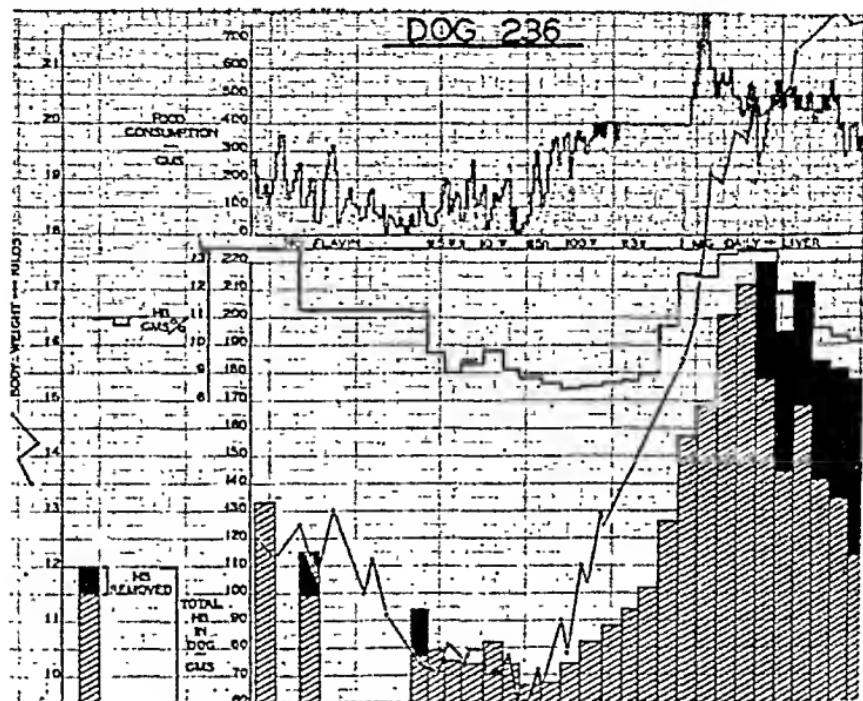


FIG. 2. Experimental history of Dog 236. The supplements of riboflavin are given as γ per kilo of body weight per day. "3" refers to 3 mg. of riboflavin daily. "1 mg. daily + liver" refers to 1 mg. of crystalline riboflavin plus 8 gm. of 1:20 liver concentrate daily.

The record of Dog 252 follows the same trend as that of Dog 251. This animal, however, responded poorly to the lower levels of riboflavin feeding. Even after 2 weeks on 20 γ per kilo daily, the hemoglobin remained at 5.9 gm. per 100 cc. of blood. When 30 γ of riboflavin were fed, there was an immediate and rapid response in hemoglobin production. During the first 6 weeks on 30 γ of riboflavin the average weekly hemoglobin made was 12.4 gm. Growth was also stimulated and a weight gain of 4100 gm. resulted. After this growth, body weight and hemoglobin rose slowly to plateaus at levels of 15.75 kilos and 11.8 gm. per 100 cc. of blood respectively.

Dog 253 also gave a picture very similar to that described in detail for Dog 251 and, unlike Dog 252, gave a better response to each level of riboflavin. During the period of depletion of riboflavin, however, this animal was not bled to the same extent as its litter mates, which would account not only for the milder anemia shown but also for the increased production of hemoglobin on the various levels of riboflavin. After 89 days on the experiment the dog had muscular spasms and collapsed. 5 mg. of riboflavin were injected and again a remarkably rapid recovery was observed.
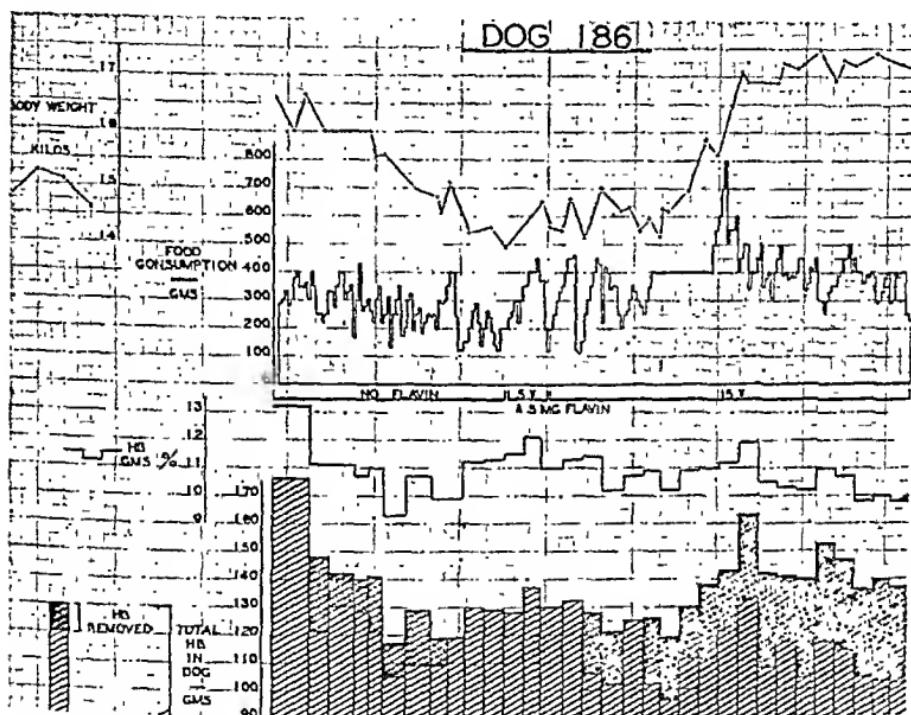
Fig. 3. Experimental history of an adult dog (No. 186). The supplements of riboflavin are given as γ per kilo of body weight per day.

Both Dogs 254 and 238 gave a typical deficiency picture during the period of riboflavin depletion. Death occurred after 10 weeks on the experiment. Evidence of a riboflavin deficiency was found on necropsy (5).

Dog 236 (Fig. 2) showed no response in hemoglobin per cent at any of the levels of riboflavin fed. Only after 8 gm. of 1:20 liver concentrate were mixed with the synthetic diet did the hemoglobin per cent rise to the normal level. However, on the assumption that 8 per cent of the dog's weight comprised the total blood volume of the animal, the dog responded typically to all lower levels of riboflavin. The hemoglobin made, as calculated, is shown in Fig. 4. But the response in hemoglobin made to

the feeding of 100 γ per kilo daily and 3 mg. daily of riboflavin was no better than that made by the other animals on the 30 γ per kilo daily level. When 1:20 liver concentrate was added to the diet, the total hemoglobin increased rapidly. It is necessary, therefore, to emphasize two facts. First, this animal was 4 months old at the beginning of the experiment and
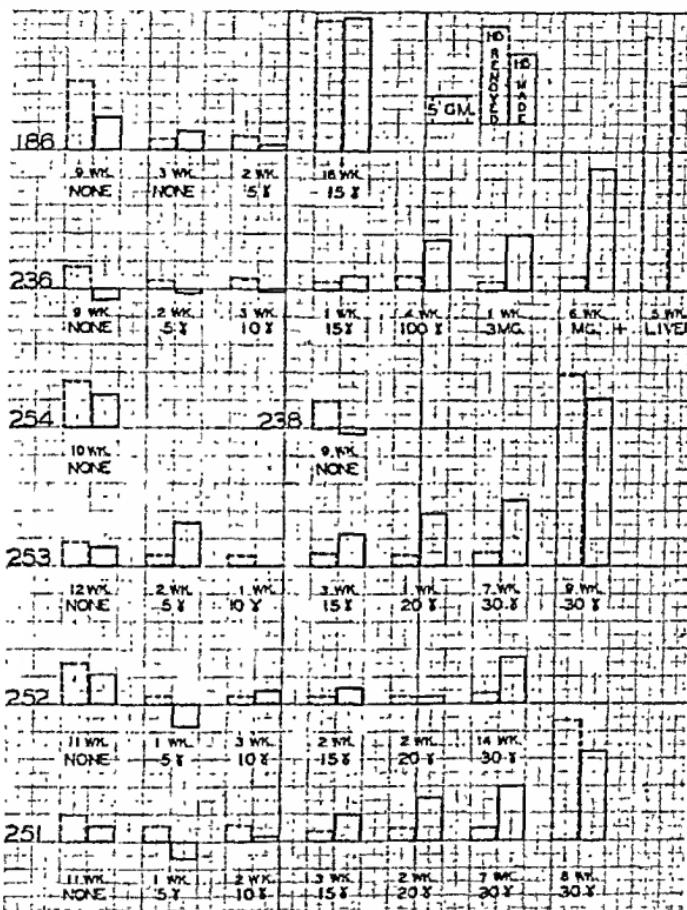
FIG. 4. Hemoglobin production during various levels of riboflavin feeding. The supplements of riboflavin are given as γ per kilo of body weight per day. "3 mg." refers to 3 mg. of riboflavin daily. "1 mg. + liver" refers to 1 mg. of crystalline riboflavin plus 8 gm. of 1:20 liver concentrate daily. The hemoglobin production and removal are expressed as a weekly average.

had previously been on a choline-deficient diet but had shown no outward signs of the deficiency. Second, the response to the liver concentrate powder is over and above that expected on the basis of the riboflavin content itself.

The results obtained in an adult dog, No. 186, differ from those pre-

viously described for the growing dogs. In 2 weeks without riboflavin the hemoglobin dropped from 13.2 to 11.1 gm. per 100 cc. of blood without bleeding. During the entire period of 9 weeks without riboflavin the average weekly removal of hemoglobin was 12.4 gm. The average amount made was 6 gm. per week. In 3 additional weeks without riboflavin in which only 20 cc. samples were removed for analysis, the hemoglobin production had dropped to a weekly average of 3.6 gm. Since the body weight dropped, the hemoglobin per cent actually rose slightly, even with this poor manufacture of hemoglobin.

4 days after administration of 5γ per kilo daily of riboflavin was begun, and 108 days after the beginning of the experiment, this animal displayed a partial paralysis of the legs and 5 mg. of riboflavin were injected. This large amount of riboflavin resulted in a sharp rise in weight and in hemoglobin level. However, the average hemoglobin made for the 2 weeks on 5γ per kilo daily was only 1 gm. per week. The riboflavin was increased to 15γ and after 2 weeks phlebotomy was begun. In the 18 week period on this level of riboflavin, 424.0 gm. of hemoglobin were removed, or a weekly average of 23.9 gm. As a result of the increased and more regular food consumption, there was also an increase in body weight to slightly above the weight at the start of the experiment.

Erythrocyte Studies—Hemoglobin production may not be the only factor limiting recovery from hemorrhagic anemia, as there may also be a reduced capacity for building erythrocytes. An animal may be capable of building cells but have a limited capacity for producing hemoglobin, which would result in hypochromia. Any limitation in the capacity to manufacture cells could probably hinder the restoration of the hemoglobin to a normal level.

The normal values at the beginning of the experiment for total cell volume (hematocrit), erythrocyte count, and cell size in the dogs used were in the ranges of 33.0 to 45.3 per cent, 5.2 to 7.5 millions per c.mm., and 59.9 to 71.8 cu. microns, respectively. These values agree with those reported in the literature (10). From Table I it can be seen that the changes in total cell volume in the various periods are of the same order as those shown in the hemoglobin level. In all cases the hematocrit returned to normal when the riboflavin level was sufficient to raise the hemoglobin to normal.

The decrease in the number of erythrocytes during the period without the riboflavin supplement is also of the same order as the changes in the hemoglobin and hematocrit. The normal number of red cells was attained in each dog but one at the very low level of 5γ per kilo daily of riboflavin feeding. Dog 236 showed a secondary decrease in the number of red cells at the higher levels of riboflavin. This may be explained by the great

increase in blood volume as already postulated. During the phlebotomy the number of red cells also showed a decrease of the same order as the decrease in hemoglobin per cent.

TABLE I

Effect of Various Levels of Riboflavin Feeding on Blood Values

Dog No.		Nor-mal	Per kilo body weight per day								Per day		
			None	5γ	10γ	15γ	20γ	30γ	30γ	100γ	3 mg.	1 mg.*	1 mg.*
251	Hb	13.1	6.8	6.5	7.6	8.3	9.7	12.8	9.38†				
	Ht	38.0	18.8	20.2	24.7	27.3	34.3	40.0	28.6				
	R.b.c.	5.3	3.7	4.4	5.4	5.7	5.9	5.6	3.8				
	M.c.v.	71.8	50.7	45.9	45.7	47.9	58.2	71.5	75.2				
252	Hb	10.7	6.5	5.8	6.0	6.1	5.9	11.8					
	Ht	33.0	20.3	22.7	23.2	21.8	22.0	37.2					
	R.b.c.	5.2	4.5	5.5	5.4	5.5	4.9	6.5					
	M.c.v.	63.5	45.1	41.3	42.8	39.6	44.9	57.2					
253	Hb	13.0	9.41	10.2	10.2	10.7	10.7	13.9	11.6†				
	Ht	43.8	32.8	36.0	35.9	35.5		44.6	41.0				
	R.b.c.	6.3	6.7	6.6	7.2	7.0		6.7	5.6				
	M.c.v.	69.5	48.9	54.5	49.9	50.7		66.5	73.2				
236	Hb	13.5	9.7	9.3	8.8	8.6				8.7	9.0	13.4	10.1†
	Ht	45.3	26.9	31.7	32.0	30.2				30.3	32.0	44.5	34.7
	R.b.c.	6.6	5.2	6.6	6.1	5.1				4.8	4.8	6.5	6.0
	M.c.v.	68.7	50.1	48.0	51.5	59.2				63.1	66.7	68.4	57.8
186	Hb	13.2	9.9	10.9		9.8†							
	Ht	43.9	34.1	36.0		33.6							
	R.b.c.	6.8	6.0	6.0		4.9							
	M.c.v.	64.6	56.8	60.0		68.5							
254	Hb	12.3	6.3										
	Ht	41.4	21.4										
	R.b.c.	6.9	4.6										
	M.c.v.	60.0	46.5										
238	Hb	11.1	6.5										
	Ht	36.5	23.7										
	R.b.c.	5.8	4.1										
	M.c.v.	62.9	57.8										

Hb represents blood hemoglobin in gm. per cent; ht, hematocrit in per cent; r.b.c., erythrocyte count in millions per c.mm.; m.c.v., erythrocyte volume in cu. microns.

* Plus 8 gm. of 1:20 liver concentrate daily.

† Phlebotomy.

The most striking changes were observed in the size of the red cells. There was a reduction in the size of the erythrocytes during the low levels of riboflavin feeding. As with the hematocrit, the red cells were of normal size when the riboflavin fed was sufficient to raise the hemoglobin to normal. Strangely enough, the red cells were of normal size during phlebotomy, even

though there was a reduction in the hemoglobin, hematocrit, and number of cells. Dog 236 produced cells only 85 per cent of the normal size during phlebotomy. This dog, however, was bled to a greater extent than the other dogs. These results are contrary to the reports found in the literature (10–14), which describe a decrease in the size of the cells during any great production of blood.

DISCUSSION

All the dogs displayed the usual symptoms of a riboflavin deficiency, but, in addition, one of the earliest changes observed was the failure to regenerate blood with phlebotomy. These experiments also indicate a definite decrease in food consumption in a riboflavin deficiency. The food consumed was sufficient only to maintain the body weight in the younger dogs (Nos. 251 to 254). The larger dogs (Nos. 186 and 236) showed a loss in body weight in the depletion period. In Dog 186 this loss in body weight was observed while the food consumption was fairly high, whereas in Dog 236 the food intake was extremely low.

The stimulus for increased food consumption was variable at the lower levels of riboflavin feeding. 15 γ per kilo per day resulted in a noticeable increase in food consumption and a rise in body weight. 30 γ produced a greater and more regular food intake and gave excellent growth. Increasing the riboflavin to 100 γ per kilo daily resulted in a still larger food intake and a more rapid gain in weight. It should be remembered, however, that the depletion of the tissues of their riboflavin and also the consequent stunting of the growth gave the dog a larger capacity for growth than would be manifest in a normal dog on the same high level of riboflavin. The adult dog (No. 186) responded with a maximum food intake at a level of 15 γ per kilo daily, which was sufficient to restore the body weight to the initial level and to allow for a slight increase. These data suggest that 30 γ are the minimal level for food consumption and good growth in young growing dogs under the conditions of our experiment. In adult dogs 15 γ per kilo daily may suffice for good food consumption and maintenance of body weight.

Investigators who observed a mild anemia in riboflavin deficiencies made no attempt to determine this relationship quantitatively. György et al. (6) measured hemoglobin production in standardized anemic dogs and found an increase above the basal level of 28 gm. per 2 weeks with daily doses of 0.1 to 0.5 mg. per kilo. As these investigators themselves point out, their basal salmon-bread diet may be open to criticism because of its high content of riboflavin which provided 0.8 to 1.5 mg. daily. Using a highly purified synthetic ration, we were able to limit the riboflavin content to 5.7 γ per 100 gm. of ration. This ration supplemented with the

crystalline B vitamins will produce an uncomplicated riboflavin deficiency when riboflavin is not included in the supplement.

Mild anemia is produced in the absence of riboflavin and a severe anemia is readily induced with slight bleeding. The dogs cannot recover from this anemia unless riboflavin is fed and show only a slight and variable hemoglobin production below a level of 15 γ per kilo daily for growing dogs. 30 γ are necessary in growing dogs for good hemoglobin production and rapid recovery from anemia. The rate of hemoglobin production is proportional to the amount fed between the levels of 15 and 30 γ. An adult dog was able to show good hemoglobin regeneration at a level of 15 γ per kilo daily. A level of 5 γ gave an insignificant amount of hemoglobin production.

These results suggest that in growing dogs there is a competitive need for riboflavin for growth and hemoglobin production. While in an adult dog 15 γ were sufficient for good hemoglobin regeneration, the added burden of growth decreased considerably the hematopoietic response of young dogs to this same level. It is unlikely that the dog shows a preferential use of the riboflavin at the low levels, for 15 γ give both a noticeable gain in weight and hematopoietic response, and higher levels of riboflavin show a proportional increase in both growth and hemoglobin regeneration.

The strain of phlebotomy is borne by the adult dog at the level of 15 γ per kilo daily, and the hemoglobin regenerated is equal to the amount removed, maintaining a normal hemoglobin level. The hematopoietic response in the younger dogs on 30 γ per kilo daily was not able to keep pace with the blood removed. At the same time there was a drop in body weight with the induction of phlebotomy. The body weight quickly reached a plateau at about 1 kilo below the level attained before phlebotomy was begun. Thereafter the body weight was maintained at the same level. This indicates that 30 γ are a marginal level and do not satisfy the increased requirement when the animal is put under any great strain of blood formation. Again we observe no preferential use of riboflavin and find the animal meets the competitive needs of weight gain and hemoglobin regeneration by impartially limiting both activities.

Dog 236 was able to maintain good growth during phlebotomy, owing to the higher riboflavin intake. The drop in hemoglobin is probably due to purely physiological reasons, the amount of blood removed being greater than the limit of the dog's capacity to regenerate it in 1 week. This dog manufactured 1.76 gm. of hemoglobin per kilo of body weight as compared to 1.67 for Dog 251 and 1.5 for Dog 186. Dog 253 demonstrated a greater hematopoietic ability throughout and produced an average of 1.91 gm. of hemoglobin per kilo of body weight during phlebotomy.

It is advisable to consider the matter of decreased food consumption

during the depletion period and to satisfy ourselves that the low food intake is not a factor in the development of the anemia. Fasting experiments have been studied in Whipple's laboratory (15). A standard anemic dog fed an optimum dose of iron with sugar and fat but no protein produced 40 to 50 gm. of new hemoglobin each week for several weeks. "Obviously [there is] a rather extensive shift of important protein material from various body depots into red cells" (15). In our experiments, anemia developed, even though the food consumed was sufficient to maintain body weight in the young dogs. The anemia is, therefore, a direct result of a riboflavin deficiency.

Studies of the red blood cells give an indication of the type of anemia produced and may perhaps offer an insight into the rôle played by riboflavin in the regeneration of blood. During the depletion period the anemia produced is of the microcytic, hypochromic type. There was also a decrease in the number of cells and, while the rate of return to normal of the hemoglobin, hematocrit, and mean cell volume is proportional to the level of riboflavin fed, the red cell count was readily restored to normal at the low level of 5 γ per kilo daily. During phlebotomy with riboflavin administration there is again a decrease in hemoglobin, hematocrit, and erythrocyte count, but the size of the erythrocytes, surprisingly, is normal. Reports in the literature of hemorrhagic anemias and of conditions in which there is an increased production of erythrocytes describe a decreased cell size (10). An inescapable suggestion from our data is that riboflavin plays some rôle in determining the size of new red blood cells formed.

The development of a satisfactory method for the determination of riboflavin has stimulated investigations of the distribution of this vitamin in various tissues. The riboflavin level of the blood (0.5 γ (16) per 100 cc. of whole blood) and the flavin-nucleotide levels of the cells and plasma are fairly constant (17). Relatively large amounts are found in the liver. It is doubtful, however, that this organ serves as a storage depot for riboflavin. Intake of large amounts of riboflavin does not increase the riboflavin content of the liver to any appreciable extent (18). Klein and Kohn (17) found a synthesis both *in vitro* and *in vivo* of flavin-adenine dinucleotide from riboflavin, in human blood cells. Although the dinucleotide occurs in plasma, its level in the plasma is not affected by incubation with riboflavin. A deficiency of riboflavin causes a decrease in the blood of riboflavin values (19).

The fundamental action of riboflavin in living tissue is to take part in enzyme systems which regulate cellular oxidations: carbohydrate and fat metabolism. The necessary participation in, and the direct influence of, riboflavin on growth and body weight gain is, therefore, obvious. Riboflavin may bear an important relation to amino acid metabolism, since

THE STABILITY OF DIPHOSPHOPYRIDINE NUCLEOTIDE IN RAT TISSUES

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Several publications have appeared in recent years which have dealt with the stability of pyridine nucleotides in macerated animal tissues (1–4). These studies have shown that such nucleotides are rapidly destroyed after maceration of the tissues. One of the steps leading to the disappearance of the physiological properties of pyridine nucleotides is a hydrolysis of the linkage between the nicotinamide and ribose parts of the molecule (3).

The stability of pyridine nucleotides after death of the animal in unbroken tissues has apparently not been investigated. Such a study should have a twofold interest. First, it would test the reliability of methods previously used in the preparation of tissue extracts for the determination of their pyridine nucleotide content; second, it would indicate the effect of cell organization on the stability of these nucleotides and, by inference, of other cell constituents of like structure.

The present study has been confined to the stability in several rat tissues of diphosphopyridine nucleotide (DPN), for which a method of determination has previously been published (5).

Methods

Albino rats were killed by a blow on the head, the time of death being noted. The organs to be studied were excised and kept in a small covered dish either at room temperature or at 6°. At various intervals a piece weighing 100 to 200 mg. was cut off, weighed on a torsion balance, and plunged into a centrifuge tube containing 3 to 5 cc. of water in a boiling water bath, the time of immersion being noted. The tube was kept in the boiling water bath for 3 minutes, cooled, and the tissue homogenized thoroughly by means of a thin glass rod. After centrifugation the supernatant was decanted into a graduated centrifuge tube or cylinder. The precipitate was stirred up with 3 to 5 cc. of water, the mixture kept in the boiling water bath for 3 minutes with stirring, centrifuged, and the supernatant added to the first. The volume of the combined extracts was noted. An aliquot of the extract was then used as the source of DPN in the "arsenate system" previously described (5) within 6 hours of the time of death of the animal.

The rates of evolution of CO_2 obtained in the presence of tissue extracts

were compared at frequent intervals with those obtained with solutions of DPN. The samples of DPN used in these calibration experiments were prepared either by the method of Williamson and Green (6), which yielded preparations containing approximately 40 per cent of DPN, or by a method previously published (7) by which 90 to 100 per cent pure preparations were obtained.[1] The purity of each sample of DPN was established by means of at least three of the following criteria: phosphorus analysis, manometric determination of dihydrogenation with dithionite (9), manometric determination of total hydrogenation of the pyridine nucleus with Pd and H_2 (10), increase in absorption at 340 mu on dithionite reduction, and comparison in the arsenate system with preparations of known purity. (For details of these procedures, see (11).)

TABLE I

Effect of Varying Modes of Extraction on Amount of DPN Extracted from Dog Blood

Samples of approximately 2 cc. of oxalated blood were pipetted into 15 cc. centrifuge tubes. The accurate volumes were determined by weighing the pipette before and after emptying, and from the specific gravity of the blood. 5 cc. of water at room temperature or 100°, as indicated below, were added to the tubes and the mixtures kept in a boiling water bath for 3 minutes with stirring. After cooling, the mixtures were centrifuged and the supernatants transferred to 25 cc. volumetric flasks. In some cases fresh 5 cc. portions of water at room temperature or 100° were added to the precipitates and the above procedure repeated. The final extracts were diluted to 25 cc. and aliquots used as source of DPN in the arsenate system.

| Sample No. | 1 | 2 | 3 | 4 | 5 |
No. of extractions	1	2	3	1	2
Initial temperature of extractant, °C.	Room	Room	Room	100	100
DPN per cc. blood, γ	25	36.5	35	33.5	33

The validity of the method of tissue extraction as described above has been tested with suspensions of sea urchin eggs (12) and dog blood. In parallel experiments it was found that the maximum amount of DPN was obtained by one extraction when the extractant was at 100° at the time of addition of the material to be extracted, by two extractions when it was at room temperature. Further increase in the number of extractions did

[1] Attempts to submit samples of DPN, obtained by Williamson and Green's method, to further purification by means of Cu_2Cl_2 precipitation have been only partially successful. While products of 90 to 95 per cent purity could be obtained, the yield in this step was only 5.5 to 7.5 per cent as compared with yields of approximately 60 per cent in our method of isolation. This conforms with the findings of Schlenk (8) that cuprous chloride precipitation is incomplete when applied to purified preparations of DPN.

not increase the amount of extractable DPN.   A representative experiment is shown in Table I.   Under these conditions, 95 to 100 per cent recovery of DPN, when added to blood before heat treatment, was obtained.   Table II contains the results of one such experiment.

Determination of the dry and of the fat-free dry weights of the tissues used was carried out in some instances, according to the method of Lowry and Hastings (13).   When either of these bases was used to express the concentration of DPN in rat organs, the standard deviation of individual results from the mean was the same as when concentrations were expressed in terms of fresh weight.   The latter basis was consequently chosen in the results to be presented.

Table II

Recovery of DPN in Preparation of Blood Extracts

Sample 1—Approximately 2 cc. of blood were extracted as described for Sample 2, Table I, except that the extracts were collected in a graduated centrifuge tube and the total volume was noted.

Sample 2—The procedure was identical except that to the first extractant 0.197 cc. of a DPN solution, containing approximately 0.1 mg. of DPN per cc., was added.

Sample 3—0.394 cc. of the DPN solution was diluted in a graduated centrifuge tube.

0.394 cc. of all extracts was used as source of DPN in the arsenate system. Specific gravity of the blood, 1.054.

Sample No.	1	2	3
Weight of blood, gm.	2.090	2.075	
Volume of mixed extracts, cc.	9.8	10.5	9.2
	γ	γ	γ
DPN in 0.394 cc. extract	1.87	2.55	1.93
" " total supernatant	46.5	68.0	45.0
" " supernatant due to blood		45.5	
" per cc. blood	23.5	23.1	
Recovery of added DPN, %		98	

EXPERIMENTAL

Rat Liver—The results of six experiments of the type described above are shown in Fig. 1.   In all cases there is a rapid decrease in extractable DPN during the first few minutes.   The rate of disappearance then decreases sharply, and after about 20 minutes a stable value is reached.   In the six experiments shown in Fig. 1, consisting of fifteen determinations, the average value with standard deviation for all results between 20 and 70 minutes after death is $756 \pm 20 \ \gamma$ of DPN per gm. of fresh liver.   These experiments include two in which the liver sample was kept at room temperature rather than at 6°.   The same result is obtained in these experiments; the average value over the same period, exclusive of the experiments at room temperature, is $759 \pm 16 \ \gamma$ of DPN per gm. of fresh liver.

The rate of disappearance of the apparent DPN content has been measured in the hope that by extrapolation one may be able to obtain the amount of DPN present in liver at the time of death. A study of Fig. 1 shows that such an extrapolation is at best very hazardous and cannot be carried out with any great certainty. The curve seems to intersect the ordinate in the neighborhood of 1050 γ of DPN per gm. of fresh liver, a value of the same order of magnitude as that reported by Axelrod and Elvehjem (14).
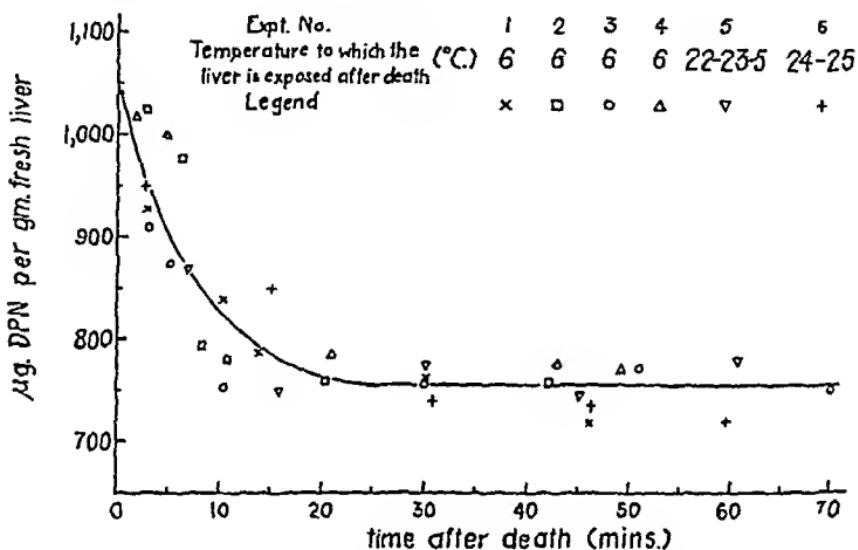

Fig. 1. The decrease in extractable DPN from rat liver as a function of time elapsed after death of the animal.

Possible Explanations for Shape of DPN Disappearance Curve in Liver

Presence of Factor in Liver Extract, Other Than DPN, Which Activates Arsenate System—The shape of the disappearance curve might be explained by assuming that there is present some other factor which is stable to the action of intracellular and extracellular enzymes of liver, while DPN is decomposed completely within approximately 20 minutes. The true DPN content would then be the difference between the initial (extrapolated) and the final stable value, or roughly 300 γ of DPN per gm. of fresh liver.

The experiment shown in Table III is presented as evidence against this explanation. It is well known that pyridine nucleotides in their oxidized form are unstable in alkaline solution, whereas their dihydro forms are easily destroyed by acid. It is also assumed that liver extracts contain a factor such as that postulated above which is not affected by oxidation in alkaline solution or reduction in acid solution. After inactivation of pyridine nucleotides by one of these procedures any residual activity of a liver

extract so treated would then be due to the mediation in the arsenate system by this factor. The results in Table III show, however, that both procedures destroy practically completely the activity of a formerly active liver extract.

The temperature-velocity coefficient of the arsenate system when catalyzed by a solution of DPN has been found to be $\mu = 16,700$ between 22.5–38° (11). This value is presumably characteristic for the DPN-catalyzed oxidation-reduction in the arsenate system, since it is the reaction limiting the rate of CO_2 production. Small variations in the concentrations of enzyme, substrate, and other components have no effect on this rate (5, 11). The same value for μ is obtained when two liver extracts, prepared

TABLE III

Evidence against Presence in Liver Extracts of Factor Other Than DPN Which Activates Arsenate System

Extract 1—10 cc. of an extract prepared from rat liver, 2 minutes after death of the animal, as described in the text.

Extract 2—To 4.0 cc. of Extract 1, 0.4 cc. of 6×10^{-5} M $K_3Fe(CN)_6$ and 0.1 cc. of N NaOH were added; pH of mixture 11.30. The mixture was heated in a boiling water bath for 30 minutes, cooled, and neutralized to phenol red.

Extract 3—To 4.0 cc. of Extract 1, some solid $Na_2S_2O_4$ and 0.1 cc. of N HCl were added; pH of mixture 1.23. The mixture was kept at room temperature for 30 minutes, aerated for 30 minutes, and neutralized to phenol red.

DPN—Solution containing approximately 5.5 γ per cc.

The above solutions, in the combinations indicated below, served as source of DPN in the arsenate system.

Flask No.	1	2	3	4	5	6
DPN, cc.	0.136			0.136		0.136
Extract No.		1	2	2	3	3
" cc.		0.197	0.30	0.30	0.30	0.30
CO_2 evolved per hr., c.mm.	38	64.5	8	47	14	28.5

approximately 3 and 30 minutes after the death of the rat, are used as the source of DPN. This indicates again that no limiting substance other than DPN is involved in the catalysis of the arsenate system.

These findings do not exclude unequivocally the presence in liver extract of an activating factor such as that postulated above. However, should such a factor exist, it shows properties of stability at varying pH and state of oxidation and a temperature-velocity coefficient identical with DPN. At present no other class of compounds is known to exhibit these properties. Possible decomposition products of DPN (nicotinic acid, nicotinamide, nicotinamide nucleoside[2]) are inactive in the arsenate system.

[2] The sample of nicotinamide nucleoside used was obtained through the courtesy of Dr. Fritz Schlenk.

Presence of Factor in Liver Extracts Which Enhances Stimulation of Arsenate System Due to DPN—In a later section it will be shown that extracts of other tissues, prepared in the same way, do not show any decay in the DPN content of these tissues over periods as long as 90 minutes. The difference between these other tissues and liver could be explained by assuming a very labile factor to be present in liver, which increases the CO_2 evolution in the arsenate system owing to the actual DPN content of the extracts. Once this activator has been destroyed, the steady value is indicative of the true DPN content of liver.

The experiment reported in Table III presents evidence against this assumption. When oxidized DPN contained in a liver extract is inactivated by alkali, addition of a solution of DPN to such an extract results in a rate of CO_2 evolution (Flask 4) which is the sum of the rates due to

TABLE IV

DPN Content of Rat Tissues

Tissue	Cerebral cortex	Thigh muscle	Kidney cortex	Liver*
DPN per gm. fresh tissue, γ...	203 ± 2	551 ± 24	533 ± 29	1050
" " " cells, γ..........	271	614	886	1345
No. of determinations.........	6	17	14	
t_1, min.......................	3.25	2.42	3.00	
t_2 "	40.00	90.75	93.50	

The relative amount of intracellular phase in the tissues was calculated from the data of Manery and Hastings (16).

t_1 = time after death of the animal when the first extract was obtained; t_2 = time after death when the last extract was obtained.

* The value reported for liver is that derived by extrapolating all of the results of the experiments with liver to zero time after death.

DPN solution (Flask 1) and to the extract (Flask 3). The same combination of flasks, when a reduced and acid-inactivated extract is used, results in an inhibition (Flask 1 + 5, more than Flask 6) which is probably due to decomposition products of dithionite still present in the extract. In no case, however, is the rate due to DPN plus extract more than the sum of the individual rates, which should be expected if the liver extracts contain a factor which acts as an activator in the presence of DPN.

Further evidence against such an assumption can be adduced. Thus, when both pure DPN and a liver extract prepared either within 2 minutes or more than an hour after death are added to the arsenate system, the rate of CO_2 production is the sum of the individual rates obtained with DPN and extracts singly. Moreover, the rate of CO_2 evolution in the arsenate system is proportional to the amount of "early" or "late" liver extract just as it is to the amount of pure DPN added to the system.

Reversible Inactivation of DPN—Lennerstrand (15) has shown that in the presence of apozymase or of muscle tissue DPN is inactivated reversibly. It was consequently thought that the disappearance of DPN in rat liver may be due to reversible inactivation caused by oxygen lack, acid accumulation, or both. However, liver slices incubated at 6° in 41 mM $NaHCO_3$ with a gas phase of either 5 per cent CO_2:95 per cent N_2 or 5 per cent CO_2: 95 per cent O_2 showed the same DPN content as slices exposed to air at 6° for the same length of time.

DPN Content of Rat Skeletal Muscle, Cerebral Cortex, and Kidney Cortex— Extracts of these organs were prepared and their DPN content determined, as described for liver. In all three tissues the value for the concentration of DPN thus obtained is the same whether tissue samples were taken shortly, or a considerable time, after death of the animal. In other words, these tissues show no disappearance of apparent DPN content. The average values and standard deviations found for all the tissues studied are given in Table IV.

<center>DISCUSSION</center>

The concentrations of DPN in the four tissues studied are presented in Table IV both in micrograms per gm. of whole tissue and per gm. of cell material. Since the DPN present in blood is known to be confined to the red (14) and white cells (11) and to be absent from plasma, the assumption appears justified that in tissues DPN is likewise an intracellular constituent. On this basis, the concentration of DPN in the rat tissues studied increases in the following order: cerebral cortex, skeletal muscle, kidney cortex, liver.

The destruction of DPN by biological materials has so far been studied only in macerates of yeast (15), almonds (17), and animal tissues (1-4), and a high rate of inactivation has been found in all cases. While DPN contained in a liver mince is inactivated very rapidly and almost completely, the present report shows that the rate of inactivation in an intact piece of liver is much lower and becomes zero at a stage when approximately 75 per cent of the original DPN remains unimpaired.

These facts suggest a relation between the DPN content of a tissue and the state of the tissue cells. In the intact cell, under physiological conditions of oxygen supply and in the proper ionic environment, DPN seems to be protected against the enzymes (nucleosidases, phosphatases) which hydrolyze the DPN molecule to smaller units inactive in the arsenate system. Once the cell is deprived of its physiological milieu, DPN can be attacked by these enzymes. It is very likely that surface phenomena play a rôle in this connection, since inactivation is favored by procedures (homogenizing, freezing and thawing) which tend to rupture the cell wall.

The fact that no disappearance of DPN in other organs can be detected

over a considerable period of time may be due either to an extremely rapid decay to a stable value during the first few minutes after death, or to a greater stability of the cells of these tissues to the unphysiological conditions prevailing after death of the animal.

Throughout this paper the words "apparent" or "extractable" DPN have been used advisedly. So far, the only criterion for "quantitative" extraction of a tissue constituent is a 100 per cent recovery of this constituent when added to the material to be extracted. While this rule may be justified when one is dealing with compounds not at all, or very loosely, associated with proteins, such as pyridine nucleotides, there is no evidence for its universal validity. Thus Westenbrink *et al.* (18) have shown that added diphosphothiamine is bound to tissue proteins more loosely than that originally present in the tissue. This conforms with the experience that, while DPN can be extracted from sea urchin eggs with 90 to 100 per cent recoveries under a great variety of conditions (12), there is no relation between the absolute amount of diphosphothiamine extracted and the degree of recovery from the same material except under sharply defined conditions (19). Should better criteria be evolved in the future, many values now accepted for the concentrations of such cellular constituents may have to undergo revision.

SUMMARY

The concentration of diphosphopyridine nucleotide (DPN) is determined in unbroken material of rat liver, cerebral cortex, kidney cortex, and skeletal muscle at various intervals after the death of the animal.

The DPN content of all tissues except liver remains constant over the period investigated (40 minutes for brain, 90 minutes for kidney and muscle). Cerebral cortex contains $203 \pm 2 \gamma$, kidney cortex $533 \pm 29 \gamma$, thigh muscle $551 \pm 24 \gamma$ of DPN per gm. of fresh tissue.

In liver, the initial value of about 1050 γ of DPN per gm. decreases sharply with time at first. After 20 minutes it becomes stable at a level of $756 \pm 20 \gamma$ of DPN per gm.

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THE DIET OF HENS AND THE VITAMIN A POTENCY OF THEIR EGGS

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Provision of vitamin A for poultry is of great practical importance, affecting health, egg production, and hatchability. The larger proportion of the vitamin usually comes from plant sources in the form of carotene, and numerous studies have shown that the vitamin A potency of the egg is related to the intake of the vitamin or of carotene, or of both, by the hen. So far as the authors are aware, no comparisons have been made with carotene and vitamin A as sole sources of potency, to determine the form and quantity of vitamin in the egg. It is well known that the color of egg yolk varies considerably, but though the total carotenoid fluctuates, the carotene in the egg yolk remains at a very low level. Strain (1) has reported traces of carotene and appreciable quantities of xanthophylls, including some cryptoxanthin, in eggs from hens on a diet containing 20 per cent of fresh alfalfa. Similar results are noted by Peterson, Hughes, and Payne (2). These reports and our own observations do not agree with the view (3) that the ratio of carotene to xanthophylls is fairly constant in yolks, regardless of the depth of color of the yolk. On the contrary, while the xanthophyll level is highly variable, that of the carotene is below 10 γ per yolk.

Because the factors influencing utilization of carotene by man are not clearly understood, it became of interest to determine whether the hen was capable of effecting an efficient conversion, so that eggs could be evaluated in terms of the vitamin itself, rather than as the provitamin, carotene. Such information bears directly on an adequate diet for the hen, and the nutritional value of the egg.

Procedure

Comparisons were made between vitamin A in the form of a shark liver oil concentrate and carotene administered in these forms: as dehydrated carrot powder, as dried alfalfa, and as crystalline carotene dissolved in ethyl laurate. Capsules containing the desired quantities of vitamin A or carotene in these forms were administered orally.

An analysis was made weekly of the carotene content of the carrot powder and of the alfalfa. During the period of feeding the change was slight, but necessitated small corrections in the supplement. A supply of carotene

was divided into small samples, each sealed in a separate ampule *in vacuo.*
Each week an ampule was opened and a weighed quantity of carotene was
dissolved in ethyl laurate. This was dispensed into capsules which were
stored at 0° until needed. No sample was stored more than 5 days. The
computation of the international units was made for the carotene on the
regular basis; 1 I.U. is equivalent to 0.6 mg. of pure β-carotene.

The shark liver oil was estimated spectroscopically to have a potency of
50,000 I.U. per gm. Some question may arise as to the desirability of using
the factor $2000 \times E_{1\,cm.}^{1\%}$ to obtain the potency in international units.
However, the factor 2000 is in regular trade use for estimation of potencies
in the fish reduction industry. A check, toward the end of the experiment,
gave the following result: 0.398 gm. of oil dissolved in 50 ml. of isopropanol,
diluted 1:25, gave a concentration of 0.0318 gm. of oil per 100 cc.

$$E_{1\,cm.}^{1\%} = \frac{0.760}{0.0318} = 24$$

Multiplying by the factor 2000, we find that the shark liver oil potency is
48,000 I.U. per gm. The error therefore is not over 4 per cent, owing to
change in potency during the course of the experiments. All stocks of
vitamin A supplements were kept under carbon dioxide gas and refrigera-
tion.

The hens were housed individually in all-metal, wire-floored laying bat-
teries and given food and water *ad libitum.* The composition of the basal
vitamin A-deficient hen diet is given in Table I. Throughout the tests egg
production remained at a satisfactory rate of 50 per cent, or more, and the
hens were maintained in good condition. The experiments may be grouped
into five time periods in which the hens received the basal diet and supple-
ments as follows:

Period I—The first period was preliminary for depletion of carotenoid
pigment reserves in the hens. Vitamin A from cod liver oil was mixed in
the diet in sufficient quantity to provide approximately 500 I.U. daily.
Twelve hens were used in this period, which lasted for 30 days.

Period II—Carrots, alfalfa, or carotene in ethyl laurate was administered
by capsule in amounts to provide 750 I.U. daily, for 6 days per week. The
period lasted for 28 days. Each supplement was fed to a group of four hens.
Analyses of the supplements are given in Table II.

Period III—Vitamin A was fed to all the hens as shark liver oil by cap-
sule at 750 I.U. daily for 6 days per week. The period lasted 23 days.

Period IV—Carotene in ethyl laurate and shark liver oil were adminis-
tered by capsule in amounts to provide 1667 I.U. daily, for 6 days per week.
The period lasted 28 days. Each supplement was fed to six hens.

Period V—A second group of laying hens was kept for 30 days on the

basal diet supplemented with cod liver oil to provide approximately 500 I.U. per day. These hens were then given only the basal diet for 17 days, after which period they were given 750 I.U. each in the form of dried alfalfa (Table II) or of shark liver oil, six times per week, for a period of 30 days.

TABLE I

Composition of Vitamin A-Deficient Diets

Ingredients	Hen diet	Chick diet
Sardine fish-meal, ether-extracted, *gm*.	15.0	15.0
Dried whey, *gm*.	2.0	
Brewers' yeast, *gm*.	3.0	7.5
Wheat bran, *gm*.	10.0	10.0
Polished rice, ground, *gm*.	62.0	63.0
Oyster shell, " "	2.0	0.5
Salt, *gm*.	0.5	1.0
Bone-meal, *gm*.	2.0	
Manganese sulfate, *gm*.	0.05	0.05
Ox bile salts, *gm*.	0.1	0.4
Choline chloride, *gm*.	0.2	0.2
Wesson oil, *gm*.	2.0	2.0
Wheat germ oil, *gm*.	1.0	
Vitamin E distillate,* *gm*.		0.002
Totals, *gm*.	99.85	99.652
Vitamin D, *A. O. A. C. units*†	100	50
" K, *mg*.‡	5	5

* A distillate containing 25 per cent of natural mixed tocopherols.

† Activated animal sterols (delsterol) free of vitamin A.

‡ In the form of tetrasodium 2-methyl-1,4-naphthohydroquinonediphosphoric acid ester.

TABLE II

Analyses of Supplements for Carotene

Period No.	Supplement	Carotene	
		γ per gm.	
II	Carrots	580, 565, 507, 495	4 successive wks.
	Alfalfa	234, 221, 245, 210	4 " "
V	"	245	

Chemical Analyses of Eggs. Periods I to V—At least 2 weeks were allowed to insure that the diet was exerting its full effect and, customarily, four eggs from each group were then taken for a given analysis as representative of that group. Although the color of the yolk varied greatly, from

was divided into small samples, each sealed in a separate ampule *in vacuo*. Each week an ampule was opened and a weighed quantity of carotene was dissolved in ethyl laurate. This was dispensed into capsules which were stored at 0° until needed. No sample was stored more than 5 days. The computation of the international units was made for the carotene on the regular basis; 1 I.U. is equivalent to 0.6 mg. of pure β-carotene.

The shark liver oil was estimated spectroscopically to have a potency of 50,000 I.U. per gm. Some question may arise as to the desirability of using the factor 2000 $\times$ $E_{1\,cm.}^{1\%}$ to obtain the potency in international units. However, the factor 2000 is in regular trade use for estimation of potencies in the fish reduction industry. A check, toward the end of the experiment, gave the following result: 0.398 gm. of oil dissolved in 50 ml. of isopropanol, diluted 1:25, gave a concentration of 0.0318 gm. of oil per 100 cc.

$$E_{1\,cm.}^{1\%} = \frac{0.760}{0.0318} = 24$$

Multiplying by the factor 2000, we find that the shark liver oil potency is 48,000 I.U. per gm. The error therefore is not over 4 per cent, owing to change in potency during the course of the experiments. All stocks of vitamin A supplements were kept under carbon dioxide gas and refrigeration.

The hens were housed individually in all-metal, wire-floored laying batteries and given food and water *ad libitum*. The composition of the basal vitamin A-deficient hen diet is given in Table I. Throughout the tests egg production remained at a satisfactory rate of 50 per cent, or more, and the hens were maintained in good condition. The experiments may be grouped into five time periods in which the hens received the basal diet and supplements as follows:

Period I—The first period was preliminary for depletion of carotenoid pigment reserves in the hens. Vitamin A from cod liver oil was mixed in the diet in sufficient quantity to provide approximately 500 I.U. daily. Twelve hens were used in this period, which lasted for 30 days.

Period II—Carrots, alfalfa, or carotene in ethyl laurate was administered by capsule in amounts to provide 750 I.U. daily, for 6 days per week. The period lasted for 28 days. Each supplement was fed to a group of four hens. Analyses of the supplements are given in Table II.

Period III—Vitamin A was fed to all the hens as shark liver oil by capsule at 750 I.U. daily for 6 days per week. The period lasted 23 days.

Period IV—Carotene in ethyl laurate and shark liver oil were administered by capsule in amounts to provide 1667 I.U. daily, for 6 days per week. The period lasted 28 days. Each supplement was fed to six hens.

Period V—A second group of laying hens was kept for 30 days on the

ments of laying hens the vegetable sources can be regarded as equal in efficiency to fish oil sources of vitamin A activity.

SUMMARY

Carotene is efficiently converted into vitamin A by the hen.   Equivalent practical dietary levels of vitamin A potency, in the form of carotene or of vitamin A, lead to an equivalent deposition in the egg of vitamin A potency which is almost exclusively in the form of true vitamin A.

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THE COLLOID OSMOTIC PRESSURE OF MIXTURES OF PROTEIN AND THYMUS NUCLEATE

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The sodium salt of thymus nucleic acid is a highly polymerized substance of molecular weight in the neighborhood of 1×10^6 (10, 12, 13).[1] At a concentration of 0.2 to 1.0 per cent, its aqueous solutions are very viscous and reveal intense streaming birefringence (5, 10, 13). The viscosity of these solutions is of the non-Newtonian type; *i.e.*, the capillary viscosity is a function of the velocity gradient for low values of the latter (5). The nucleate particles are quite asymmetric in shape; values of 300 for the ratio of long to short axes of a rod-like particle (10) and of 450 mμ for the length of such a particle (13) have been estimated. When to aqueous solutions of thymus nucleate, salts, amino acids, or proteins are added, the viscosity and the intensity of birefringence decrease, and the extent of this decrease is determined by the concentration and by the nature of the added substance (5). Among the simpler molecules, substances containing the guanidinium ion such as guanidine hydrochloride or arginine monohydrochloride are particularly effective.[2] When placed on a molar basis, solutions of purified proteins have by far the greatest effect upon the specific properties of the thymus nucleate of any of the substances studied.[3] The effect produced by addition of any one of the purified materials is very nearly instantaneous and the new and lowered values observed for the nucleate properties in the mixtures are maintained sensibly constant over a period of many hours. No dialyzable products of the nucleate are ever found.

It is evident that a profound interaction between the thymus nucleate and the added substances must take place whereby the specific properties,

[1] Kahler, H., personal communication.

[2] Myosin is a protein the particles of which are highly elongated in shape (2). Edsall and Mehl (3) have shown that many substances can effect a reduction of the viscosity and streaming birefringence of solutions of this protein. It is curious that the properties of both myosin and of thymus nucleate are most sensitive to the action of guanidine salts.

[3] The relative viscosity of sodium thymus nucleate in 0.5 per cent aqueous solution drops from about 50 (at 16.0 cm. of H_2O pressure and 30°) to about 20 in a dilute solution of horse serum albumin (2.46 mg. of N per cc.). Addition of guanidine hydrochloride to the aqueous nucleate solution to make a concentration of 10^{-5} M of the salt reduces the relative viscosity to about 40. The effects produced by these and other substances may therefore be of a considerable order of magnitude.

which depend upon the shape of the particles of the former, are reduced. In order to study this interaction further, and to see whether the osmotic properties of the nucleate are also affected, the osmotic pressure of non-diffusible mixtures of nucleate and protein was determined over a wide range of varying ratios of the two components.

<div align="center">EXPERIMENTAL</div>

The sodium thymus nucleate was Preparation 2 which had been extensively employed and characterized in earlier studies (6, 4). Aqueous solutions of this preparation show intense streaming birefringence at a concentration as low as 0.1 per cent. It was dissolved in distilled water to 1.0 per cent concentration; *the solutions were prepared fresh every day.* The protein solutions consisted of fresh horse serum (5.6 per cent protein) and an aqueous solution of horse serum albumin crystallized three times which had been brought to pH 7.0 by addition of dilute sodium hydroxide. The protein concentration of the albumin solution was 4.0 per cent. Sodium chloride was added to the albumin solution to 0.9 per cent concentration. Three different samples of serum were used. The pH of all the original solutions used for the mixing was very nearly the same, 6.9 to 7.1, and there was no observable change in the pH after mixing. The colloid osmotic pressure was determined by the method of Hepp (1, 8), with a parlodion membrane.

The outside fluid (in the capillary) had approximately the same sodium chloride concentration to begin with as that of the inside (test) solution. The protein solutions were diluted with distilled water, and thymus nucleate solutions with 0.9 per cent sodium chloride. The osmotic pressure readings for the protein solutions alone and for the protein-thymus nucleate mixtures became constant within half an hour after being placed in the osmometer and remained so to ±1 per cent for at least 12 hours thereafter. The readings for the pure thymus nucleate solutions appeared to reach equilibrium values within half an hour after being placed in the osmometer; but on further standing there was a slight but steady drop in the osmotic pressure at the rate of about 1 per cent per hour. After standing for 12 hours at 22°, solutions of thymus nucleate generally decreased about 10 per cent in osmotic pressure from the value for the first half hour, and about 20 per cent in viscosity. The outside solutions gave a negative test in every case for thymus nucleate (phosphorus). The slow spontaneous decrease in the specific physical properties of aqueous solutions of thymus nucleate has been described before (5, 7). It is for this reason that the solutions of the nucleate were prepared fresh every day. The various mixtures were prepared just before they were placed in the osmometer. All readings of the osmotic pressure of the various solutions described in the present paper were taken 60 minutes after the solutions were placed in the osmometer.

The measurements were performed on mixtures of thymus nucleate and serum or albumin in varying proportions and on control solutions of protein or of thymus nucleate each mixed with the appropriate volume of water or of 0.9 per cent saline. The ratios of protein to nucleate in the mixtures were so selected that a range of 1.4 to 22.4 mg. of protein per mg. of nucleate

TABLE I

Colloid Osmotic Pressure of Mixtures of Sodium Thymus Nucleate with Protein

Temperature $23° \pm 0.5°$. The outside solutions were made up in the same salt concentrations as the inside solutions before the latter were tested. All readings were made after 60 minutes of equilibration in the osmometer.

Mixture	Protein-nucleate ratio	Colloid osmotic pressure
	mg. per mg.	*mm. H_2O*
Undiluted horse serum (5.6% protein)...............		281 ± 3
2.0 cc. serum + 0.5 cc. H_2O		218 ± 4
2.0 " 0.9% NaCl + 0.5 cc. 1% nucleate............		24 ± 1
2.0 " serum + 0.5 cc. 1% nucleate.................	22.4	214 ± 4
2.0 " " + 1.0 " H_2O..............		181 ± 3
2.0 " 0.9% NaCl + 1.0 cc. 1% nucleate............		46 ± 2
2.0 " serum + 1.0 cc. 1% nucleate.................	11.2	185 ± 4
2.0 " " + 2.0 " H_2O..........................		144 ± 3
2.0 " 0.9% NaCl + 2.0 cc. 1% nucleate............		71 ± 1
2.0 " serum + 2.0 cc. 1% nucleate.................	5.6	145 ± 5
2.0 " " + 4.0 " H_2O..........................		103 ± 2
2.0 " 0.9% NaCl + 4.0 cc. 1% nucleate............		91 ± 2
2.0 " serum + 4.0 cc. 1% nucleate......	2.8	111 ± 5
2.0 " " + 8.0 " H_2O.........		56 ± 1
2.0 " 0.9% NaCl + 8.0 cc. 1% nucleate............		121 ± 2
2.0 " serum + 8.0 cc. 1% nucleate.......	1.4	85 ± 2
Undiluted horse serum albumin (4.0%)		172 ± 4
2.0 cc. serum albumin + 1.0 cc. H_2O... ...		114 ± 2
2.0 " 0.9% NaCl + 1.0 cc. 1% nucleate............		46 ± 2
2.0 " serum albumin + 1.0 cc. 1% nucleate....... .	8.0	117 ± 3
2.0 " " " + 2.0 " H_2O...... ...		86 ± 5
2.0 " 0.9% NaCl + 2.0 cc. 1% nucleate............		71 ± 1
2.0 " serum albumin + 2.0 cc. 1% nucleate.	4.0	86 ± 2
2.0 " " " + 4.0 " H_2O		63 ± 2
2.0 " 0.9% NaCl + 4.0 cc. 1% nucleate......... ...		91 ± 2
2.0 " scrum albumin + 4.0 cc. 1% nucleate..... ...	2.0	69 ± 3

was studied. Several readings were made on each solution and the variations are given with the data in Table I.

Inspection of the data in Table I reveals the striking fact that although solutions of protein and of nucleate separately exert a definite osmotic pressure, mixtures of the two exert an osmotic pressure which with one exception is practically the same as that of the protein alone. The excep-

tion is the mixture in which the ratio of serum to nucleate was very low. In the other cases it would appear that the nucleate in mixtures containing protein simply did not contribute to the osmotic pressure of the mixture, at least in so far as the sensitivity of the instrument could detect.   In view of the close concordance of the values for the mixtures and for the protein controls it is unlikely that the values of the former are attributable to a simultaneous fall in the osmotic pressure of both components in the mixtures.   In all the mixtures in which the osmotic pressure was practically that of the protein component, the streaming birefringence had very nearly disappeared.   In the case of the mixture in which the protein-nucleate ratio was lowest and the osmotic pressure of 85 mm. of H_2O was intermediate in value between that of 56 for the diluted serum control and 121 for the diluted nucleate control, the streaming birefringence of the mixture, although lower in intensity than that of the latter control, was still clearly evident.   It is obvious that a distinct interaction occurs between protein and thymus nucleate which affects not only the shape factors of the latter but its osmotic properties as well.[4]   In no case was there any evidence of nucleic acid phosphorus in the fluid outside the membrane.

Study of the effect of dilution on the serum and on the nucleate solutions revealed that the osmotic pressure is a linear function of the concentration, at least within the concentration range observed (Table I).   The successive dilutions involved reductions in concentration of 20, 33.3, 50, 66.7, and 80 per cent, respectively.   The osmotic pressure for either protein or nucleate decreased with dilution in nearly this order.   The linear relation in the case of sodium thymus nucleate was observed by Hammarsten (7).   The osmotic pressure of the albumin solutions also appears to be linear in the concentration.

<center>DISCUSSION</center>

The practical identity of the values for osmotic pressure in the various mixtures of protein and nucleate with those observed for the respective protein solutions alone (Table I) indicates that in these mixtures the

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[4] The scrum of male rabbits contains the active, heat-labile thymonucleodepolymerase (5).   Mixtures of equal volumes of this serum and 1.0 per cent thymus nucleate have a relative viscosity of 1.2 after 6 hours of incubation at 30° and 16.0 cm. of H_2O pressure in contrast with a relative viscosity of 51.3 for a mixture of equal volumes of water and 1.0 per cent thymus nucleate.   The osmotic pressure of the mixture of serum and nucleate at this stage is 160 mm. of H_2O; that of a mixture of equal volumes of serum and water is 151 mm. of H_2O.   The outside solution showed no nucleic acid phosphorus.   Mixtures of aqueous extracts of mouse lymph nodes with thymus nucleate (in which the relative viscosity reaches a value of 1.2 in 15 minutes) when dialyzed in cellophane bags for 6 hours against distilled water did not yield diffusible nucleic acid phosphorus.

thymus nucleate is osmotically ineffective. The loss in osmotic activity of the nucleate in the presence of protein may be tentatively interpreted on the basis that in the presence of protein the former is converted to an undissociated, or nearly undissociated form. The term undissociated as used here does not imply that the phosphate groups of the nucleate are not fully ionized but that the sodium ions of the nucleate are so closely associated with the very large polyvalent ion that they are osmotically ineffective. The forces of attraction may be expected to be considerable in the neighborhood of the large polyvalent ions, and indeed Hammarsten (7) and Kern (9) demonstrated that thymus nucleate and the polyacrylic acids under certain conditions could render smaller ions osmotically inactive.[5] This phenomenon, referred to frequently as the Hammarsten effect, has been described by Kern as an "osmotic buffering," in analogy with the familiar buffering of weak acids and salts. The extreme case, in which few or none of the sodium ions of the thymus nucleate is osmotically effective, appears to occur when protein is present. On this basis, the distribution of sodium chloride across the membrane, which is controlled by the dissociation of the nucleate, becomes equalized and the contribution of the nucleate-salt system to the osmotic pressure of the mixture drops out. The protein-salt system, on the other hand, appears to be entirely or nearly entirely unaffected in the mixtures.

Stenhagen and Teorell (11) studied the electrophoretic behavior of a mixture of serum albumin and thymus nucleate in the weight ratio of about 1.6:1. Part of the nucleate migrated with the protein at about the mobility of the latter alone; the other part of the nucleate migrated independently at the mobility of the pure component in buffer alone. The fraction of the nucleate which was affected by the protein and migrated with it must have borne a net electrical charge considerably lower than that of the free material in buffer alone, and this is consistent with the explanation of the results of the osmotic pressure measurements described in this paper.

The interpretation which has been tentatively advanced for the loss in the osmotic activity of sodium thymus nucleate in the presence of protein has been based upon the suppression in dissociation of the former. Perhaps the most obvious way in which such an effect could be produced would be by way of aggregation of the nucleate particles either with or without a previous depolymerization, although there is as yet no independent evidence to support this concept. However, such an aggregation leading to more globular masses of particles would also explain the loss in the properties of streaming birefringence and structural viscosity of the nucleate, for thereby

[5] Hammarsten (7) represents the sodium ions under these conditions as "hiding" within the framework of the giant thymus nucleate ion.

the extreme asymmetric shape upon which these properties depend would be reduced.

SUMMARY

The colloid osmotic pressure of mixtures in varying proportions of serum or of serum albumin and sodium thymus nucleate in the presence of sodium chloride was determined.   Controls of similar dilutions of the separate components were likewise studied.   Except for the mixture containing the lowest ratio of protein to nucleate, wherein the osmotic pressure was intermediate between those of the two components, the osmotic pressure of all of the mixtures studied was nearly identical with that of the protein component.   The osmotic activity of the nucleate was practically absent in these mixtures.   The effect upon the osmotic pressure of the nucleate in the protein mixtures was tentatively interpreted as being ascribable to a suppression of the dissociation of the nucleate accompanied by probable aggregation of the latter.

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DETERMINATION OF AMINO ACIDS BY THE SOLUBILITY PRODUCT METHOD

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A quantitative knowledge of the amino acid composition of a protein is essential to an understanding of its chemical structure. Pending the procurement of complete data, Bergmann and Niemann (1, 2) employed the available information on the amino acid composition of the proteins to formulate numerical rules governing the stoichiometry of the amino acid residues within the protein molecule. It is recognized that the ultimate proof of the adequacy of these rules, or the possibility of their expansion, depends on the development and application of more general and more accurate methods for the determination of amino acid constituents. To this end the present studies have been directed.

The experimental procedure for the determination of amino acids in protein hydrolysates by the solubility product method is described in this communication. The work to date is largely developmental. In view of the fact that further work along these lines is not possible at the present time, the procedure is being described, since it may be useful for the determination of amino acids and also, in some cases, for the determination of other classes of compounds which form sparingly soluble dissociable salts. The experimental techniques outlined below have also been found suitable for the establishment of the purity of organic compounds by phase rule solubility curves.

The general development of the solubility product method (3–5) has been discussed in a recent review (6). The following brief summary of the present formulation of the method, as it applies to glycine and l-leucine, is limited to the considerations essential to an understanding of the experimental manipulations.

In the determination of leucine, for example, with an aromatic sulfonic acid reagent, two aliquots of an amino acid solution or a protein hydrolysate are taken, each aliquot containing A mM of leucine. A sample of a sparingly soluble sulfonic acid salt of leucine is added to the first aliquot (Solution 1, Fig. 1) and, after equilibrium has been reached at $0°$, measurement is made of the amount of the salt (S_1 mM) which has gone into solution. The leucine salt has dissolved to the point of saturation against the common ion effect of the A mM of leucine initially present. To the second aliquot (Solution 2, Fig. 1), R mM of the free sulfonic acid (or sodium salt) are

added and the solubility (S_2 mμ) of the leucine salt is also determined in this solution at the same temperature. In this second case the common ion effect of the added sulfonic acid further lowers the solubility of the salt.

As the binary salt dissolves, it contributes equimolar quantities of leucine and sulfonic acid to the solution. The total amount of sulfonic acid present in the first solution is, therefore, S_1 mμ and the total amount of leucine is $(A + S_1)$ mμ. The product of these two quantities is the solubility product $K_1 = S_1(A + S_1)$. At equilibrium in the second solution, the total amount of sulfonic acid is $(R + S_2)$ mμ, the total amount of leucine is $(A + S_2)$ mμ,

Fig. 1. The solubility product method

and the solubility product is represented by the expression, $K_2 = (R + S_2)(A + S_2)$. If the solubility product is a constant under these conditions, as has been found to be the case in the determination of leucine, the two K values may be set equal to each other and the resulting equation solved for A in terms of S_1, S_2, and R (Equation I, Fig. 1). If the values of K are not identical, as has been found to be the case in determinations of glycine, a factor F (Equation II, Fig. 1) may be introduced to account for small deviations if they are reproducible.

The manipulations described below are, therefore, directed toward the accurate determination of the three quantities, S_1, S_2, and R. The method for leucine and glycine is described in detail in order to illustrate this type of approach to protein constituent analysis.

Technique of Solubility Measurement

The method requires the measurement of the solubility of a given solid phase in two aliquots of the solution under analysis. In the present procedure, a weighed amount of the amino acid salt is added to the solution, the mixture is stirred slowly until equilibrium is attained at 0°, and the two phases are separated without temperature change by centrifugation at 0° through sintered glass, as described by Ing and Bergmann (5). Determination of the true weight of the undissolved solid phase requires measurement of the small amount of solution adhering to the crystals and the filter. This is accomplished by weighing the filter assembly before and after drying.

Apparatus

The flask for the solution and solid phase, the sintered glass filter, and the centrifuge tube are of Pyrex glass (Fig. 2, a). The apparatus illustrated is designed for use with about 3 cc. of solvent. The dimensions may be modified to meet other volume requirements. For the filtration of most crystalline organic compounds the sintered glass plates (about 2 mm. thick) may be Ace porosity E or Corning porosity "fine." The neck of each flask should fit loosely in the constricted top of the filter. The glass bead in the flask is present to increase the efficiency of stirring.

The centrifuge tubes are 150 × 25 mm. test-tubes suitably constricted as indicated in Fig. 2, a. The rubber washer on the tip of the filter is cut from 10 mm. bore, heavy wall vacuum tubing and is sufficiently large to insure against glass to glass contact between the filter tip and the centrifuge tube during centrifugation. The size of the bottom washer, which is made from rubber tubing, varies with each tube, the thickness being such that the filter and flask are held firmly when the rubber stopper is inserted. The wire loop is added to aid in the removal of the filter after centrifugation.

For weighing the filter and flask, a standard 100 × 25 mm., light weight, soft glass weighing bottle is used. For each weighing bottle, filter, flask, and bead, a counterpoise is prepared from a similar 100 × 25 mm. bottle containing small glass beads (not lead shot). Each set and its counterpoise are kept as a unit to avoid retaring. The centrifuge tubes and accessories, however, may be used interchangeably. The units are conveniently handled in groups of six. Inclined racks made from monel screen (Fig. 3, b) are used for handling the weighing bottles and for storage of the counterpoises near the balance. The sintered plate on which samples of the filtrate are weighed and dried is larger (Fig. 2, c) than that of the filter and may be of medium porosity. It fits a 50 × 25 mm. weighing bottle and is supplied

with a corresponding counterpoise. The racks for the smaller weighing bottles are cut lower than are those for the larger weighing bottles shown
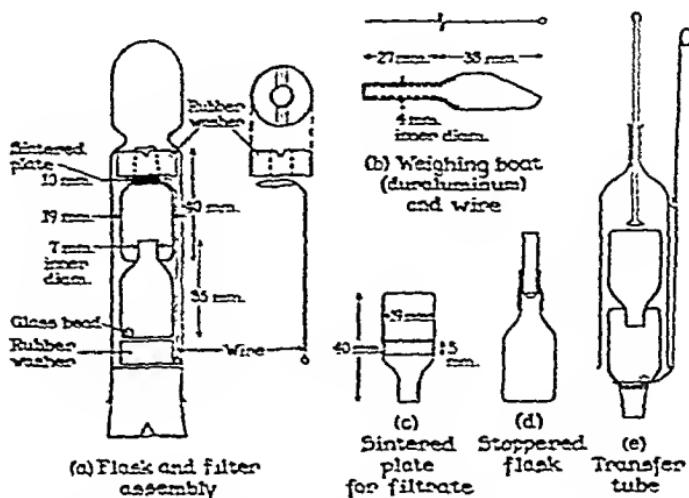

FIG. 2. Apparatus for use in the solubility product method
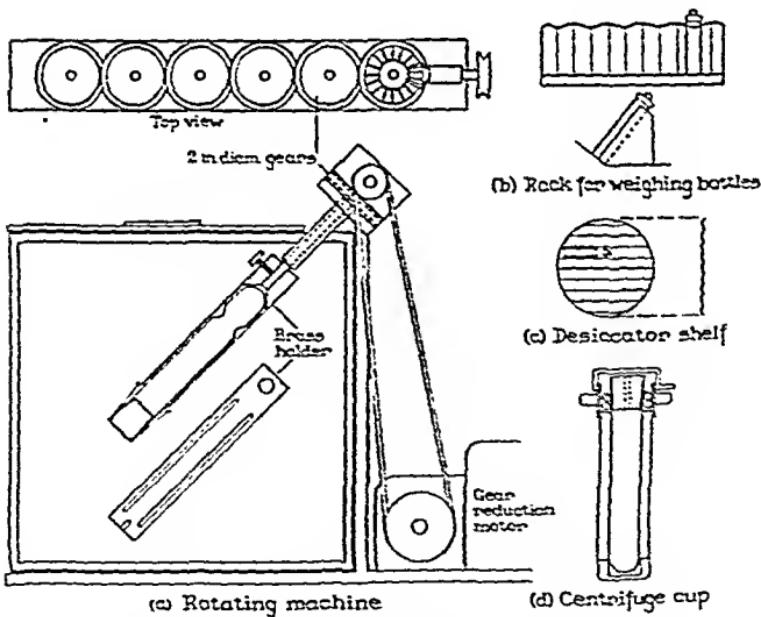
FIG. 3. Apparatus for use in the solubility product method

in Fig. 3, *b*. The tops of the bottles should project above the rack to facilitate handling with forceps.

The weighing boat for solid samples (Fig. 2, *b*) may be turned out from duralumin. It is slightly flattened on the bottom to prevent rolling on the

balance pan and has a hollow neck. A counterpoise is made from sheet aluminum. A wire is conveniently employed in some cases to loosen crystals which fail to fall readily through the neck of the boat. The wire has a separate counterpoise. The stopper shown in Fig. 2, d is placed on the flask in order to prevent evaporation after the solution has been pipetted into the flask. It is formed from the lower half of a small test-tube and a rubber tubing collar. The transfer tube, used during the removal of the filter and flask from the centrifuge tube, is of Pyrex glass.

The solutions are efficiently and smoothly stirred by rotating the tubes in an inclined position (Fig. 3, a). The angle on the rotating machine is 45° and the speed about 150 R.P.M. This method of stirring has proved to be preferable to the rocking type of agitation employed in earlier work (5). The tubes are securely held on the shafts by detachable brass holders. The sides of the brass tubes are slotted to permit circulation of water around the glass. The bath in which the assembly rotates is filled with chipped ice and water. In this laboratory the rotors are operated in a refrigerated room maintained at 2°, and uninsulated baths (15 × 10 × 10 inch aquaria with covers) maintain a temperature of 0° for 24 hours. For operation at other temperatures, suitable thermostats and, perhaps, insulated baths would be required.

When equilibrium between solid phase and solution has been attained, the tubes are inverted and centrifuged in International No. 365A centrifuge cups (Fig. 3, d). A standard 150 × 25 mm. test-tube is used as a mold for freezing a sleeve of ice to fit the centrifuge tubes. A fixed rubber ring in the bottom of the cup and a collar on the top of the tube serve to center it during the freezing. A thin lining of rubber dam is placed on the inner wall of each cup. A rubber cap (1¾ inches) closes the top of the cup to prevent entrance of the alcohol-ice-water freezing mixture. Each set of six cups is racked in a 6 inch wire basket in a freezing bath (11 × 11 × 9 inch aquarium). The desiccator shelf, also shown in Fig. 3, c, may be made of monel screen to fit a 250 mm. Pyrex vacuum desiccator and to accommodate six 100 × 25 mm. and six 50 × 25 mm. weighing bottles.

For the scale of operations in the present experiments, a semimicro balance is desirable. In the analyses summarized in Tables I and II all weighings were made to 0.01 mg. and the experimental error of the amino acid determinations was thus maintained below 2 per cent. Weighings to 0.1 mg. on a chainomatic macro balance give an average experimental error of 5 per cent.

Procedure

Cleaning of Apparatus—Crucible tongs with rubber tubing on the tips are used to handle the glassware. Acetone (1 to 2 cc.) is drawn through each filter by suction and while the filter is still moist with acetone it is

immersed upright in boiling water, removed, and the hot water sucked through the plate. Three washings with hot water are followed by a final rinse with acetone. The flasks are cleaned similarly with acetone and hot water. They are drained with the aid of suction by inversion over a 1-hole stopper (10 mm. bore) on a 1 liter suction flask.

The bead from each filter is tipped out into the corresponding flask and the flask and filter are wiped with a linen cloth and placed in the weighing bottle. The inner surfaces of the weighing bottles are washed occasionally. At intervals of a dozen or so filtrations, the filters are cleaned with hot concentrated sulfuric and nitric acid.

The cleaned assemblies are dried in a vacuum desiccator under the conditions to be used in the determination for which they are intended (see below). The sintered plates for samples of the filtrate are similarly cleaned and dried.

Weighing—The weighing bottles are wiped carefully soon after removal from the desiccator and are placed beside the balance for 30 minutes.[1] With forceps (7 inch, with rubber tubing on the tips) a set of six bottles is put into the balance case on the left and the corresponding counterpoise bottles on the right. (With a set of six, three analyses are run simultaneously.) The weights are taken after the glassware has been in the case for 15 minutes. With practice in wiping and handling, the weight of a given assembly can be reproduced to 0.02 mg. when put through all the manipulations, including centrifugation.[2] The use of counterpoise bottles of the same surface area is especially essential in this method, since there is an interval of 48 hours between the first and final weighings. Reproducible results are impossible if brass weights are used in view of changes in temperature and humidity.

For convenience in handling, the flasks and filters are now transferred from the weighing bottles to 50 cc. beakers. Aliquots of the solutions under analysis are pipetted into the flasks, which are then stoppered. Care is taken to touch off the pipette on the liquid surface and to remove it without depositing any drops of solution in the neck of the flask.

The appropriate quantity of sulfonic acid reagent, R, is added to Solution

[1] As outlined here, the bottles are wiped before each weighing. A careful, reproducible technique is adopted, an ethanol-moistened portion of a linen cloth or a chamois being used first, followed by a dry section. An alternative procedure is to eliminate the wipings and arrange the operations throughout the determination so that the bottles are touched only by forceps or cloth and not by hand. In this case the weighings can be made 15 minutes after removal from the desiccator.

[2] To new filters and flasks, 3 cc. samples of distilled water are added and the assemblies centrifuged, dried, and reweighed, to ascertain whether they maintain constant weight. Filters may show a small loss during the first two or three centrifugations. It is not essential for the filters to remain constant during the cleaning process, which is more severe treatment than they receive in centrifugation.

2 of each determination. The approximate quantity of solid may be weighed into the duralumin boat on a macro balance prior to the semimicro weighing. It is convenient to have two counterpoises for the boat, one for each balance. The boat is lifted from the balance pan with forceps and the transfer of the sample is made by inserting the hollow neck of the boat into the unstoppered flask to a level a little above the liquid. In most cases, tapping the boat will cause the solid to fall through the hollow neck into the solution. The insertion and removal of the neck of the boat must be made without depositing any crystals on the upper walls of the flask where they may fail to be brought into solution. If the use of the wire plunger is required to effect the transfer, it should be placed on the balance pan during the first as well as the second weighing of the boat. After transfer of the sample, the flask is restoppered, and the boat reweighed.[3] The boat is cleaned with water and acetone before being used to weigh a different compound. The flasks to which the reagent has been added are rotated by hand to bring the reagent completely into solution. The amino acid salt to be used as solid phase is weighed into Solution 1 and Solution 2 for each determination in the same manner. A few crystals adhering to the neck of the flask are not a source of error in this case, since the salt is present in excess.

Attainment of Equilibrium—A set of centrifuge tubes is numbered with wax pencil to correspond to the filters being used. The apparatus is assembled as shown in Fig. 2, *a*. The filter is handled with a cloth and the flask is carefully wiped if it has been touched during the pipetting and weighing of samples. In this and in all subsequent parts of the procedure care is taken not to jar the solution into the neck of the flask. If it is difficult to insert the rubber stoppers smoothly, they may be lubricated with glycerol and the excess carefully removed with a towel. It is essential that the rubber stoppers be inserted firmly into the centrifuge tubes to prevent water from entering the assembly under the slightly negative pressure developed when it is cooled to 0°.

In the cold room a brass holder is slipped over each centrifuge tube and the latter is held in position by a rubber band over the two brass clips and through the notches of the rubber stopper. The holder is securely fastened on the shaft of the rotor by a thumb-screw. The tubes are usually rotated in the ice bath overnight. Pure solutions of amino acid salts come to equilibrium with this technique in about 1 hour. Well clarified protein hydrolysates may require about 5 hours. The adequacy of overnight rotation may be checked by running duplicate solubilities (S_1), the second tube being taken off after 40 hours. The two values should be the same.

At the time the tubes are put on the rotor, the mold tubes are placed in

[3] A glass boat is not satisfactory in this procedure in view of the time required for it to attain constant weight after handling.

the centrifuge cups; the space between the wall of the tube and the wall of the cup is filled with water; and the set of six is placed in the freezing bath which is brought to an initial temperature of about $-2°$ by alcohol, ice, and water. The level of the freezing mixture is kept several mm. below the tops of the cups.

Centrifugation—Before centrifugation, the thoroughly frozen centrifuge cups are taken from the alcohol-ice bath and water at room temperature is run into the mold tubes to loosen them sufficiently for removal. The cups are subsequently allowed to stand until melting is noticeable, indicating that their temperature is no longer below $0°$. A brass holder is detached from the rotor and inverted to the centrifuging position while held in the ice bath. The glass centrifuge tube is slipped out of the brass holder and immediately into the ice sleeve of a centrifuge cup. The cups are balanced with drops of ice water and centrifuged at 1800 R.P.M. The time required for complete filtration varies with filter porosity and crystal size. In most cases 10 minutes are ample time. If the centrifuge is in a refrigerated room, the ice sleeves will maintain constant temperature in the cups for 30 or 60 minutes. At room temperature they are efficient for more than 10 minutes.

Final Weighings—After centrifugation, the glass centrifuge tubes are transferred to a suitable test-tube rack and removed from the cold room. A tube is dipped into a beaker of water at room temperature for about 40 seconds to bring it above the dew point. The stopper is removed and the neck wiped free from moisture. The filter and flask are pulled into the glass transfer tube with the aid of the wire loop and hook. After removal of the washer and wire, the filter tip is wiped and the filter and flask transferred horizontally to the corresponding weighing bottle.

With protein hydrolysates and synthetic mixtures it is essential to determine experimentally the per cent solids in the mother liquor, in order to calculate the correction to be applied for mother liquor adhering to the crystals after centrifugation.[4] For this purpose a 100 to 200 mg. sample of the filtrate from the centrifuge tube is pipetted onto one of the sintered glass plates (Fig. 2, c). The plate may be left in the 50×25 mm. weighing bottle during this operation. The amount added to the plate should not be more than it will absorb.

The flasks and filters and filtrate plates are weighed to 0.1 mg. before drying. The weights are taken 30 to 60 minutes after centrifugation. No special wiping precautions are necessary in this step. The flasks and filters

[4] In work with simple known solutions, containing only one amino acid, for example, this part of the procedure may be omitted. The small correction for mother liquor adhering to the crystals after centrifugation can be calculated readily from the loss on drying, the density of the solvent, and the approximate amount of solids known to be in solution.

and the filtrate plates are dried (usually overnight) in the same desiccator and the final weights are taken to 0.01 mg.

Drying—The exact conditions of drying must be worked out for the determination of each individual amino acid. In the determinations of leucine, with sodium 2-bromotoluene-5-sulfonate as reagent, and of glycine, with 5-nitronaphthalene-1-sulfonic acid as reagent, which are described in this communication, the filters and flasks are dried overnight at 15 mm. over $CaCl_2$-KOH at 25°. Constant weight is usually attained in 5 hours, and overnight drying provides an ample margin of safety. The leucine bromotoluenesulfonate dries to the monohydrate under these conditions and shows a slow continued loss of weight of about 0.3 per cent per 24 hours. This small loss affects the weights of the precipitates from Solutions 1 and 2 similarly and causes only negligible deviations in the calculations.

Glycine nitronaphthalenesulfonate dries to the anhydrous salt under these conditions.

In preliminary studies, drying at 100° and 1 mm. for 2 hours was found to yield the anhydrous leucine salt and to work well for determinations in which pure leucine solutions were employed. Egg albumin hydrolysates, on the other hand, cannot be brought to constant weight at 100°. The continued loss of weight may be followed for 100 hours to a point well below the known initial solid content of the hydrolysate. Similar results are obtained with the synthetic mixture employed in Experiment 5, Table II. When dried on sintered glass under a vacuum at room temperature, however, hydrolysates come to constant weight in about 3 hours.

Selection and Preparation of Reagents

The selection of suitable reagents (6–8) for the determination of amino acids by the solubility method can only be mentioned briefly here. For the determination of leucine, 2-bromotoluene-5-sulfonic acid has been proved to have the desired specificity for most analytical applications. The solubility products of the sparingly soluble amino acid salts of bromotoluenesulfonic acid in N HCl at 0° are 9×10^{-4} for *l*-leucine, 10×10^{-4} for *l*-phenylalanine, 3×10^{-4} for *l*-tryptophane, and 2×10^{-4} for *l*-histidine. In the solubility method in its present form, phenylalanine will not interfere with the leucine determination unless it is present in more than equimolar proportion to leucine. Tryptophane is rarely a potential source of interference. The solubility product of the histidine salt is that of a ternary salt, involving the square of the reagent concentration. Calculation, based on the reagent concentration in a leucine determination, shows that leucine may be estimated in the presence of several equivalents of histidine.

For the present experiments *l*-leucine was purified over the naphthalenesulfonate (4) or the bromotoluenesulfonate (7, 8). For use as solid phase,

l-leucine bromotoluenesulfonate $+ H_2O$ (mol. wt. 400.1) was prepared from purified l-leucine and purified sodium bromotoluenesulfonate[5] and was recrystallized from 20 per cent alcohol and air-dried to constant weight. Sodium bromotoluenesulfonate $+ \frac{1}{2}H_2O$ (mol. wt. 292.1) prepared as previously described (8) was recrystallized from 50 per cent alcohol and air-dried. The crystal size of the compounds should be small enough to permit convenient weighing and transfer of mg. quantities. A sample of each compound is dried to constant weight at 100° and 1 mm. to check the water of hydration or moisture content.

For the determination of glycine, 5-nitronaphthalene-1-sulfonic acid has proved applicable. The approximate solubility products of the sparingly soluble amino acid salts of nitronaphthalenesulfonic acid, determined at 0° in N HCl-methyl cellosolve (30 per cent) are 1×10^{-3} for glycine, 2×10^{-3} for l-hydroxyproline, 5×10^{-3} for l-phenylalanine, 4×10^{-5} for l-arginine, 1×10^{-4} for l-histidine, and 6×10^{-4} for l-lysine. The principal amino acids that may interfere with the glycine determination, therefore, are hydroxyproline at more than 2 equivalents and arginine at more than $1\frac{1}{2}$ equivalents. The phenylalanine, histidine, and lysine salts are relatively soluble compared to the glycine salt.

For the present experiments glycine nitronaphthalenesulfonate (mol. wt. 328.3) and nitronaphthalenesulfonic acid $+ 2H_2O$ (mol. wt. 289.3) were prepared as previously described (8).

The absence of interference by other amino acids in the solubility determination is checked first by the addition of about 5 per cent more than $(R + S_2)$ mM of sulfonic acid reagent to an aliquot of the solution under analysis. No precipitate should appear at 0°. A second check is made by determining the amino acid content of the hydrolysate after the addition of a known quantity of the amino acid under analysis (cf. Tables III and V).

In order to remove amino acids which might interfere with a given determination, preliminary precipitation with other sulfonic acid reagents may be a practical procedure in special cases. All solutions for analysis should be relatively free from inorganic cations, many of which form sulfonates of low solubility.

The purity of the amino acid, the sulfonic acid, and the salt must be established not only by the usual tests for purity, but also by the operational procedure on which the solubility method itself is based. This is done in the following manner: The amino acid salt, ar, is prepared from the purest available samples of the amino acid, a, and the reagent, r, and the salt is subjected to several recrystallizations. Two nearly saturated solutions are prepared; the first contains equimolar amounts of a and of r and the second

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[5] The direct preparation of the salt from leucine and the sodium salt of the reagent is made in the presence of 1 or more equivalents of HCl.

contains the salt, *ar*, alone. Equal aliquots of the two solutions are pipetted into flasks for solubility determinations. Equal quantities of the solid phase, *ar*, are added to each solution, and the two solutions are brought to equilibrium at 0°. To the extent that *ar* is added as solid phase in both cases, it contributes equally to both $K_{(a + r)}$ and $K_{(ar)}$ and does not interfere with the detection of differences between the solubility product of the components, *a* and *r*, and that of the salt, *ar*. If the initial components are

TABLE I

Establishment of Purity of Reagents

The amino acid and the reagent were weighed into one 25 cc. volumetric flask, and the salt into another, to give solutions of the indicated concentrations per 3 cc. Approximately 20 cc. of solvent were added to each flask and the solids dissolved by rotating the flask in a beaker of warm water. The cooled solutions were made to volume and aliquots removed for solubility determinations. The amount of solid phase added to each aliquot was 50 mg. The solutions of *a* and *r* and of *ar* are made up to be identical in every respect, including Na^+ and Cl^- concentrations. In the glycine measurements, an excess of the N HCl-methyl cellosolve (30 per cent) mixture is prepared and two 25 cc. portions taken from the same sample of the solvent.

	Leucine system		Glycine system	
	$(a + r)$*	(ar)	$(a + r)$†	(ar)
	mM	*mM*	*mM*	*mM*
Amino acid (a)..............	0.09120		0.08752	
Reagent (r).................	0.09014		0.08783	
Salt (ar)...................		0.09120‡		0.09172
Solid phase dissolved (s)......	0.00102	0.00063	0.01337	0.00911
	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$	$\times 10^{-4}$
Solubility product§ (K)........	9.34	9.37	11.34	11.30
Duplicate determination (K)...	9.38	9.36	11.27	11.31

* Sodium 2-bromotoluene-5-sulfonate = *r*. Solvent, N HCl.

† 5-Nitronaphthalene-1-sulfonic acid = *r*. Solvent, N HCl-methyl cellosolve (30 per cent).

‡ Also 0.090 mM of NaCl and 0.09 cc. of water were added per 3 cc., since *r* is the sodium salt of the sulfonic acid.

§ Calculated on the basis of moles per liter. $K_{(a + r)} = (a + s)(r + s)(1/v^2)$; $K_{(ar)} = (ar + s)^2 (1/v^2)$. v = volume in cc.

pure, the solubility product as measured on the recrystallized salt, $K_{(ar)}$, is identical with that obtained on the components, $K_{(a + r)}$. If, however, the two solubility products are not equal, the components should be subjected to further purification. An independent check on the purity of the salt is obtained by recrystallizing it to constant solubility.

The results given in Table I serve to establish the purity of the compounds used in the present leucine and glycine determinations.

Determinations of l-Leucine

The results of determinations of leucine, based on the formulation of Fig. 1 and the technique of the preceding experimental sections, are given

TABLE II

Estimation of l-Leucine by Solubility Product Method

Solid phase, l-leucine 2-bromotoluene-5-sulfonate + H_2O.

Experiment 1, no addition. In Experiment 2, 10 per cent methyl cellosolve was present per 3 cc. (in addition to leucine (solvent, N HCl); in Experiment 3, 15 per cent methyl cellosolve; in Experiment 4, arginine 31.8 mg., glutamic acid 26.4 mg., glycine 13.5 mg., and tyrosine 32.4 mg.; in Experiment 5, alanine 6.0 mg., arginine 5.0 mg., aspartic acid 12 mg., cystine 6.0 mg., glutamic acid 12 mg., glycine 6.1 mg., histidine 3.6 mg., lysine 4.0 mg., phenylalanine 12 mg., proline 12 mg., tyrosine 12 mg., and ammonia 1.6 mg.

Experiment No.	l-Leucine content per 3 cc. (A)	Reagent added (R)	Salt dissolved		Solubility product*		l-Leucine found (Equation 1, Fig. 1)
			S_1	S_2	K_1	K_2	
	mM	mM	mM	mM	$\times 10^{-4}$	$\times 10^{-4}$	per cent recovery
1	0.09160	0.07994	0.05637	0.00605	9.27	9.33	98.0
2	0.10688	0.10245	0.06380	−0.00047	12.09	12.06	100.9
3	0.11363	0.10335	0.06785	0.00278	13.68	13.73	99.1
4	0.09247	0.08827	0.06900	0.01542	12.38	12.43	98.6
5	0.09165	0.08845	0.06330	0.00915	10.90	10.93	99.1
	0.09165	0.09118	0.06388	0.00815	11.04	11.01	100.7

* K_1 and K_2 are expressed here on the basis of moles per liter, or $S_1 (A + S_1)/9$ and $(R + S_2) (A + S_2)/9$.

TABLE III

l-Leucine Content of Egg Albumin

There has not been opportunity to investigate at great length the leucine content of other proteins. Preliminary analyses have been performed, however, on hydrolysates of gelatin, collagen (cattle Achilles tendon), and silk fibroin. The results indicate that gelatin and collagen contain about 3.5 per cent l-leucine each, and that silk fibroin contains about 0.8 per cent l-leucine.

Experiment No.	Protein equivalent per 3 cc. hydrolysate	Reagent added (R)	Salt dissolved		l-Leucine found	Per cent of protein
			S_1	S_2		
	mg.	mM	mM	mM	mg.	
1	132.8	0.09278	0.06488	0.00845	12.1	9.1
2	142.4	0.09308	0.06200	0.00413	12.8	9.0
3	71.2 + 6.01 of l-leucine	0.08980	0.06000	0.00405	12.5 (6.5 from protein)	9.1

in Table II. l-Leucine has been determined in pure solutions, in synthetic mixtures of amino acids, and in hydrolysates of egg albumin (Table III). The reagent, R, is the sodium salt of 2-bromotoluene-5-sulfonic acid. The

solid phase is *l*-leucine bromotoluenesulfonate. The two solubility products in each control determination are found to be essentially equal to each other and the recoveries of leucine obtained by the application of Equation I (Fig. 1) are in a range from 97 to 101 per cent.

There has been little or no quantitative information on the leucine content of proteins. The absence of adequate analytical methods for leucine can be attributed primarily to its lack of characteristic functional groups. That the present method is capable of application to problems of protein constitution is shown by experiments with hydrolysates of egg albumin (Table III), in which the reproducibility of the method is comparable to that attained with synthetic amino acid mixtures and the recovery of pure leucine added to the hydrolysate is quantitative.

Inherent in the principle of the solubility product method of analysis and essential to its practice (4) is the fact that the accuracy of the method, in contrast to the usual gravimetric analysis, is not dependent upon the solvent power of the solution. Thus, as can be seen in Table II, although the solubility product in known leucine solutions is varied from 9.3×10^{-4} to 13.7×10^{-4}, the accuracy of the analysis is maintained. In the egg albumin hydrolysate of Experiment 1, Table III, K is 11.3×10^{-4}.

Determination of Glycine

When the method is applied to the determination of glycine, with 5-nitronaphthalene-1-sulfonic acid as reagent, a constant solubility product is not obtained. A series of results is summarized in Table IV. The addition of methyl cellosolve to these determinations is made to increase the accuracy of the experimental measurements by increasing the solubility product of the glycine salt from the value of 5×10^{-4}, found in water alone, to the value 10×10^{-4}. As will be noted in the experiments with known amounts of glycine, K_2 is greater than K_1 in each instance. The ratio of K_1 to K_2, however, is fairly constant over the relatively wide range of conditions covered by these experiments. When an average value for this ratio of 0.939 is substituted for F in Equation II, Fig. 1, the recoveries of glycine in all cases are within the range 98 to 103 per cent. The glycine content of the protein silk fibroin (Table V), determined by this method, is in agreement with the value of 43.8 per cent obtained by Bergmann and Niemann (9) with potassium trioxalatochromiate.

Data and Calculations

A data sheet on the determination of leucine in a protein hydrolysate is given in Table VI. In both the leucine and the glycine determinations the amount of solid phase added to Solution 1 is 60 to 70 mg. and to Solution 2 is 35 to 45 mg. The values for R, S_1, and S_2 are converted from mg. to mM for substitution in Equation I or II, Fig. 1.

TABLE IV

Estimation of Glycine

Solid phase, glycine 5-nitronaphthalene-1-sulfonate.

Experiment No.	Present per 3 cc. in addition to glycine (solvent, N HCl-methyl cellosolve)	Methyl cellosolve
	mg.	*per cent*
1		25
2		30
3		35
4		40
5	Glucose 21.6	30
6	Furfural 26	30
7	Arginine 10, glutamic acid 26, leucine 24, tyrosine 18	30
8	Mixture of twelve amino acids corresponding to Experiment 5, Table II	30

Experiment No.	Glycine content per 3 cc. (A)	Reagent added (R)	Solubility product*		$\frac{K_1}{K_2}$	Glycine found (Equation II, Fig. 1) F = 0.939
			K_1	K_2		
	mM	*mM*	$\times 10^{-4}$	$\times 10^{-4}$		*per cent recovery*
1	0.08817	0.08814	7.33	7.83	0.936	99.1
2	0.09137	0.08901	9.39	10.06	0.934	98.2
3	0.09197	0.08990	12.07	12.82	0.942	100.8
4	0.10515	0.11403	17.00	18.10	0.939	100.0
5	0.09015	0.09001	9.23	9.79	0.943	101.0
6	0.09123	0.08911	10.84	11.50	0.943	101.0
7	0.09047	0.08712†	12.14	12.84	0.946	102.9
8	0.08918	0.08795	13.88	14.83	0.936	98.7

* K_1 and K_2 have been calculated on the basis of moles per liter.

† The S values, to give one example, were $S_1 = 0.06867$ and $S_2 = 0.01870$.

TABLE V

Glycine Content of Silk Fibroin

There has not been opportunity to investigate at great length the glycine content of other proteins. Preliminary analyses have been performed, however, on hydrolysates of gelatin, collagen (cattle Achilles tendon), and egg albumin. The results indicate that gelatin and collagen have glycine contents of 25.5 and 26.2 per cent, respectively. These values are in agreement with the earlier values of 26.0 and 26.5 per cent found for these proteins with potassium trioxalatochromiate as reagent (3). Egg albumin was found to contain about 3.1 per cent glycine. This figure on egg albumin is not final, but indicates a glycine content considerably higher than that found by earlier methods.

Experiment No.	Protein equivalent per 3 cc. hydrolysate	Reagent added (R)	Glycine found	Per cent of protein
	mg.	*mM*	*mg.*	
1	15.84	0.08820	6.86	43.3
2	15.84	0.09132	7.03	44.4
3	15.84	0.08590	6.97	44.0
4	7.92 + 3.48 of glycine	0.08627	6.93 (3.45 from protein)	43.6

126

Preparation of Protein Hydrolysates

The egg albumin used in this investigation was prepared by the method of Kekwick and Cannan (10), and was recrystallized three times. To remove inorganic material, the preparation was denatured by heating in water, and the coagulum filtered off and washed with water until the washings gave a negative test for sulfate ion. Analysis of the filtrate and washings revealed that no nitrogen-containing substances were removed from the protein by this treatment. The denatured egg albumin thus obtained contained 0.22 per cent ash (as sulfate) and 15.4 per cent nitrogen, calculated on an ash- and moisture-free basis.

TABLE VI

Data Sheet; Determination of Leucine (Experiment 1, Table III)

	Solution 1	Solution 2	Wet weight correction factors	
			Solution 1	Solution 2
(a) Tare, mg..................	2.10	0.92	0.19	0.74
(b) Reagent (R), mg...........		27.10		
(c) Solid phase added, mg......	59.36	35.95		
(d) Weight, wet, mg............	70.5	71.3	160.5	171.4
(e) " dried, mg.........	38.05	36.28	11.84	13.35
(f) Loss on drying, "	32.5	35.0	148.7	158.0
(g) Residue, (e) − (a), mg.....			11.65	12.61
(h) Factor, (g) ÷ (f).........			0.0784	0.0798
(i) Correction, (h) × (f), mg..	2.55	2.79		
(j) Undissolved solid phase, (e) − ((a) + (i)), mg...	33.40	32.57		
(k) S, (c) − (j), mg............	25.96	3.38		
(l) " from (k), mM*..........	0.06488	0.00845		
(m) R " (b), "†		0.09278		

* Leucine bromotoluenesulfonate + H_2O, mol. wt. 400.1.

† Sodium bromotoluenesulfonate + $\frac{1}{2}H_2O$, mol. wt. 292.1.

The silk fibroin was prepared from Japanese raw white silk. The sericin was removed by repeated treatment of the silk with H_2S-papain at 40°. The resulting fibroin preparation contained 0.06 per cent ash (as sulfate) and 19.0 per cent nitrogen, calculated on an ash- and moisture-free basis.

For rapid attainment of equilibrium between solid phase and solution, hydrolysates should be as nearly humin-free as possible. Stannous chloride sometimes is useful in minimizing humin formation during hydrolysis. In the case of egg albumin, 10 gm. were hydrolyzed (oil bath at 120°) to constant NH_2-N in 30 hours by 100 cc. of 20 per cent HCl containing 2 gm. of $SnCl_2 \cdot 2H_2O$. The resulting solution was filtered through sintered glass and the major part of the HCl removed from the filtrate by repeated concentration to a sirup under reduced pressure. The solution was diluted to

about 150 cc. and treated with H_2S to remove tin. The colorless filtrate from the tin sulfides was concentrated to a sirup which was diluted to about 100 cc. with water and the protein equivalence per cc. determined by total N. For approximation of the titratable acidity of the hydrolysate solution, a 1 cc. sample was diluted to 20 cc. and titrated to phenolphthalein with 0.1 N alkali (found 1.2 N).

For the determination of leucine, an aliquot of this solution was pipetted into a 15 or 25 cc. volumetric flask and made to volume with water and N HCl to give a final acidity of about 1 N. Approximately 100 mg. of carbon (Darco S-51) were added per gm. of protein. The flask was shaken for 5 minutes and the solution filtered by gravity through paper or by pressure through sintered glass. When 3 cc. samples of the filtrate are used for each solubility measurement, 12 cc. of solution are required for a leucine determination in duplicate.

The carbon clarification is carried out immediately before the analysis is set up and removes the major part of the coloration which usually develops in hydrolysate solutions during storage. Decolorizing carbon in large enough amounts is capable of adsorbing leucine from aqueous solution. Control experiments have shown that the amount of carbon used above does not lower the leucine content of the hydrolysates. The use of carbon in considerable excess of the minimum quantity required to remove most of the humin coloration is to be avoided.

Silk fibroin (21 gm.) was satisfactorily hydrolyzed by boiling concentrated HCl (60 cc., 8 hours) without the addition of $SnCl_2$. The major part of the HCl was removed by repeated concentrations of the hydrolysate to a sirup under reduced pressure. A preliminary clarification was effected by the addition of 6 gm. of $CuCl_2$, and subsequent precipitation of the copper with H_2S. The pale yellow filtrate from the copper sulfide was concentrated *in vacuo* once more to remove HCl, and made up to a volume of 200 cc. The protein equivalence was determined by total N. In the dilution of the samples for the glycine analyses, methyl cellosolve was added to give a 30 per cent concentration. The acidity of the aqueous solution should be 1 ± 0.2 N, the 70:30 aqueous methyl cellosolve mixtures corresponding to about 0.7 N acid. Immediately before an analysis is set up, a carbon clarification is performed in the manner described in the determination of leucine. Glycine is not adsorbed by carbon to the same extent as is leucine, but, nevertheless, an excess of carbon is to be avoided because of the likelihood of introducing significant quantities of ash into the hydrolysate.

DISCUSSION

Solubility Product As Variable—The theoretical and practical considerations governing the constancy or lack of constancy of the solubility product in experiments of this type require brief explanation. In this method of

analysis there is a difference both in the amino acid concentration and the sulfonic acid concentration between Solutions 1 and 2. In addition to possible variations in activity coefficients, these concentration differences involve physical changes in the nature of the solvent. Aromatic sulfonic acids, for example, are to varying degrees surface-active agents. As their concentration in solution increases, the solvent power of the solution may be altered. For reasons of this type the change in K occurring in the glycine determination is a reflection of a general tendency for K not to remain constant in the solubility method. Although the solubility product is constant in the special case of the leucine analysis ($K_1/K_2 = 1.00$), the constancy can be proved to be the result of a compensation of several factors. The presence of NaCl lowers the solubility of leucine bromotoluenesulfonate in N HCl. In the analyses in Table II the sodium salt of the reagent is used, and the resulting presence of NaCl in Solution 2 serves to compensate in part for the increase in K due to increased sulfonic acid concentration, which otherwise would be apparent. Also serving to compensate in the same direction is the fall in the leucine concentration from ($A + S_1$) to ($A + S_2$). This represents a change in the solvent independent of activity variations which simultaneously may be involved. For example, in experiments independent of common ion effects, the addition of leucine measurably increases the solubility of glycine nitronaphthalenesulfonate, and the presence of glycine increases the solubility of leucine bromotoluenesulfonate. Tests of this type indicate qualitatively the nature of the complex variations whose summation is measured in the ratio of $K_1:K_2$. Experimentally, however, in the leucine and glycine procedures the ratio can be maintained constant. The addition of a given amount of a sulfonic acid reagent, a factor which can be controlled, induces a typical series of concentration changes which is reproduced in each determination. Mathematically, a constant $K_1:K_2$ ratio is as satisfactory as a constant solubility product.

The considerations discussed above do impose the requirement that for accurate determinations the concentration of the particular amino acid under analysis and the total acidity (1.0 ± 0.2 N) be similar to those used in Tables II and IV. On an unknown protein hydrolysate, therefore, a preliminary analysis is necessary in order to determine the concentration of hydrolysate required for precise determinations.

Stereochemical Considerations—The configuration of the amino acid salt employed as solid phase in the solubility method governs the stereochemical specificity of the determination. In the present estimations of *l*-leucine the *l* salt is employed as solid phase. Small amounts of *d*-leucine, if present, do not enter into the equilibrium. Determinations of *d*-leucine, however, have been made by use of the *d* salt as solid phase.

Solubility Curves—The semimicro experimental procedure for the meas-

urement of solubility described in this communication has also been found useful in this laboratory for the establishment of the purity of organic compounds by phase rule studies. One modification is introduced when the relationship of solubility to the amount of solid phase present is under investigation. It is desirable to bring the mixture of solvent and solid phase to 0° before stirring is initiated in order to prevent supersaturation in those points in which the amount of undissolved solid phase is only 2 or 3 mg. Solubility curves of high accuracy have been obtained by this procedure on compounds to which total N of the filtrate, or a similar analysis, was not applicable.

SUMMARY

The solubility product method of analysis described in detail in this communication makes it possible to determine glycine or leucine on a semimicro scale in protein hydrolysates, and is in principle applicable to the determination of other protein constituents.

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THE CYTOCHROME C CONTENT OF NORMAL AND NEOPLASTIC MAMMALIAN EPITHELIUM, AND ITS CORRELATION WITH BODY MASS*

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The deduction may be drawn from the findings of several investigators (2–5) that a deficiency of cytochrome c is a biochemical characteristic of malignant tumors. However, the soundness of this view may be questioned. It was founded upon a comparison of the cytochrome c content of malignant neoplasms, regardless of their origin, with that of normal rat organs, such as the heart, kidney, liver, and brain, in which malignant growths virtually never arise spontaneously. In seeking more extensive and reliable information in this problem we considered it desirable to provide data, heretofore unavailable, upon the following pertinent points: (1) the cytochrome c content of those rat tissues in which spontaneous neoplasms do develop, (2) the cytochrome c content of the tissues of man, a species which, in contrast to the rat, has a particularly high tendency towards neoplasia, and (3) the relationship of the content of cytochrome c and the factor of cellularity, since variations in the amount of inert mass, such as extracellular matrix and intracellular depot substances, could be expected in the various tissues examined.

The investigation has been confined mainly to epithelial tissues, because in the case of man such material is more frequently obtainable through surgical removal than are mesenchymal tissues and sarcomas. The cytochrome c content of neoplastic epithelium of rat and man has been found to be very low in comparison with that of normal rat epithelium from the kidney, liver, and brain. To this extent the results of previous workers have been corroborated. On the other hand, the mammary gland of the rat and the colon mucosa of man, epithelial tissues which are common sites of spontaneous neoplasms in the respective species, proved to be not significantly richer in cytochrome c than the neoplasms derived from these tissues.

means of isobutyl alcohol. Time was saved and uniformity of extraction in separatory funnels was insured by the use of a mechanically driven rocking device. This permitted the simultaneous extraction of ten separate solutions, which included one reagent blank, two standard solutions containing respectively 10 and 50 γ of phosphorus, and seven experimental solutions. Photometry was carried out in a Klett-Summerson photoelectric comparator (9), with a green filter. Under our working conditions the optimal range of the method was 6 to 60 γ of phosphorus. The reproducibility of duplicates was within 5 per cent.

EXPERIMENTAL

Concentration of Protein-Bound Phosphorus in Mammalian Tissues—
In Table I examples are provided of representative values for the concentration of protein-bound phosphorus in various types of epithelial tissues (Experiments 4a to 9) as well as in some mesenchymal tissues (Experiments 1, 2, 3, and 10), which are encountered admixed with normal and neoplastic epithelium. The analyses (P values in Table I) are expressed upon a dry rather than wet weight basis, since the water content may be altered considerably by procedures such as rinsing with Ringer's solution for the removal of mucus or in the manipulation attending surgical resection. In those instances in which comparison with values upon a wet weight basis may be of interest, such values may be calculated from the ratios of wet to dry weight (WW/WD), given in Column 5 of Table I. In such tissues as kidney cortex, liver, submaxillary gland, and colon mucosa the concentration of protein-bound phosphorus proved to be characteristic of the particular tissue, and independent of the species of origin. Hence in these cases the P values for only a single species are cited. However, significant species differences, as may be seen, were encountered in smooth muscle, lung, and brain cortex, although in the latter tissue the P values for the rat and the mouse were of similar magnitude.

It may be predicted from the data in Table I that a dilution of the dry matter of a tissue with fat (Experiments 1 and 2) or with glycogen (Experiments 6a and 6b) would result in a diminution of the P values. Conversely, an increase in the number of nuclei, and therefore presumably in the number of cells (compare Experiments 3 and 10, or Experiments 4 and 8), leads to higher values for the protein-bound phosphorus. The advantages of expressing the concentration of cytochrome c upon the basis of the quantity of protein-bound phosphorus per unit of dry weight of tissue rather than upon the basis of dry weight itself thus become obvious. By referring the cytochrome c to the P values an automatic adjustment is supplied for the variable factors of inert tissue mass and storage substance, and an approach is made to an expression of results upon the basis of relative cellu-

larity of the tissue. It is recognized that the correlation of protein-bound phosphorus and the cellular content of tissues (cellularity) is not exact, nor is it known at present whether such a correlation is applicable to all types of tissue.

TABLE I

Protein-Bound Phosphorus Content of Mammalian Tissues

Experiment No. (1)	Tissue (2)	Species (3)	No. of samples (4)	$\frac{WW^*}{WD}$ (5)	P† (6)	S.e. $\times$ 100§ $\overline{P}$ (7)
					γ per gm.‡	
1‖	Mesenteric fat	Rat	1	1.08	44	
2¶	Panniculus adiposus	"	1	1.49	370	
3a‖	Smooth muscle, stomach	Man	2	5.78	1050	±3
3b¶	" " colon	Rat	2	4.95	1660	±10
4a**	Brain cortex	Rabbit	6	5.40	1160	±4
4b**		Rat	4	4.56	1560	±5
5**	Kidney cortex	"	7	4.49	2850	±7
6a‖	Liver, glycogen-rich††	Rabbit	2	3.89	2470	±7
6b‖	" normal††	"	4	4.11	3300	±4
7a‖	Lung	Man	1	5.79	2510	
7b¶		Rat	4	4.61	3740	±7
7c‖		Rabbit	4	4.84	4850	±5
¶	Submaxillary gland	Rat	5	4.11	5610	±4
9‖	Colon mucosa‡‡	Rabbit	4	5.63	6090	±7
10¶	Lymph nodes	Rat	1	3.53	8420	

* Ratio of the wet weight to dry weight of tissue.

† Protein-bound phosphorus. In those cases in which more than one sample was analyzed the values represent the mean.

‡ Per gm. of dry weight of tissue.

§ Standard error $= \pm \sqrt{\Sigma d^2/n(n-1)}$. P = corresponding P values in Column 6.

‖ Samples from individual animals.

¶ Samples from pooled organs of several animals.

** Samples included from individual as well as pooled organs.

†† The designations "glycogen-rich" and "normal" differentiate the livers which yielded turbid filtrates from those which gave relatively clear filtrates in the analysis for cytochrome c. Reference may be made to our previous paper (6).

‡‡ The term "colon mucosa" as used by us refers to the mucous membranes of the descending colon, sigmoid, and upper rectum.

Concentration of Cytochrome c in Epithelial Tissues of Rat—Table II presents a summary of our determinations of cytochrome c in various normal epithelial tissues of the rat and in a group of epithelial neoplasms derived from the mammary gland of this species. The cytochrome c concentrations are expressed both per gm. of dry weight of tissue (C values, Column 5) and upon a protein-bound phosphorus basis, calculated from the C values

larity of the tissue.   It is recognized that the correlation of protein-bound phosphorus and the cellular content of tissues (cellularity) is not exact, nor is it known at present whether such a correlation is applicable to all types of tissue.

TABLE I

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(1)	(2)	(3)	(4)	(5)	(6)	(7)
					γ per gm.‡	
1‖	Mesenteric fat	Rat	1	1.08	44	
2¶	Panniculus adiposus	"	1	1.49	370	
3a‖	Smooth muscle, stomach	Man	2	5.78	1050	±3
3b¶	" " colon	Rat	2	4.95	1660	±10
4a**	Brain cortex	Rabbit	6	5.40	1160	±4
4b**		Rat	4	4.56	1560	±5
5**	Kidney cortex	"	7	4.49	2850	±7
6a‖	Liver, glycogen-rich††	Rabbit	2	3.89	2470	±7
6b‖	" normal††	"	4	4.11	3300	±4
7a‖	Lung	Man	1	5.79	2510	
7b¶		Rat	4	4.61	3740	±7
7c‖		Rabbit	4	4.84	4850	±5
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TABLE III

Cytochrome c Content of Epithelial Tissues in Various Species

Experiment No. (1)	Tissue (2)	Species (3)	No. of samples* (4)	$\frac{WIW}{IVD}$ (5)	Dry weight basis			Protein-bound P basis		
					C (6)	$\frac{\text{S.e.}\times100}{C}$ (7)	$\frac{C\times100}{C_{rat}}$ (8)	$\frac{C}{P}$ (9)	$\frac{\text{S.e.}\times100}{C/P}$ (10)	$\frac{(C/P)\times100}{(C/P)_{rat}}$ (11)
					γ per gm.†		per cent	γ per γ‡		per cent
1	Kidney cortex	Mouse§	2	3.73	1105	±1	77	0.339	±7	65
2		Rabbit	5	4.89	354	±7	25	0.115	±7	22
3		Dog	1	4.72	302		21	0.117		·22
4		Pig	1	4.51	271		19	0.086		16
5		Man	3	4.48	45	±11	3	0.017	±13	3
6		Horse	1	4.95	137		10	0.055		11
7	Liver‖	Mouse§	2	3.14	525	±13	87	0.144	±11	75
8		Rabbit	4	4.11	125	±20	21	0.037	±18	19
9		Horse	1	3.53	24		4	0.008		4
10	Brain cortex	Mouse§	1	4.58	479		128	0.283		117
11		Rabbit	6	5.40	252	±9	67	0.218	±8	90
12	Submaxillary gland	"	3	4.42	190	±6	50	0.033	±7	45
13	Colon mucosa	"	4	5.63	97	±15	71	0.016	±12	76
14		Man	7	6.03	39	±19	28	0.007	±19	33
15	Lung	Rabbit	4	4.84	45	±6	141	0.009	±5	157
16		Man	1	5.79	9		29	0.004		63
17	Gastrointestinal¶ adenocarcinomas	"	6	5.59	30	±25	42	0.006	±30	60

The symbols employed in the headings of the table are identical in meaning with corresponding symbols in Tables I and II. The subscript "rat" in C_{rat} and in $(C/P)_{rat}$ (Columns 8 and 11) is used to indicate that the mean C values in corresponding rat tissues, found in Column 5 of Table II, have served as standards of comparison. See the text.

* The tissues were from single individuals, except in the cases of mouse tissues and the submaxillary gland of the rabbit in which pooled samples were analyzed.

† Per gm. of tissue.

‡ Cytochrome c per γ of protein-bound phosphorus.

§ Rockefeller Institute inbred strain.

‖ All the liver samples used belonged to the group designated "normal" (see foot-note to Table I). The analysis of one rabbit liver has been omitted. This sample was treated with diastase, as described in our previous paper (6), and yielded for this species an unusually low C value of 21.

¶ This group includes two gastric and four rectal tumors.

100. In the case of the group of human adenocarcinomas (Experiment 17 in Table III), due to limitations in availability of material we had to rely

for comparison upon the Walker carcinoma of the rat (Experiment 10, Table II), whose C value was taken as 100.   Since the cellular composition of the experimental rat tumor differs considerably from that of the human neoplasms,[2] it is possible that the relative cytochrome c content of the latter (Experiment 17, Columns 8 and 11, Table III) may have been somewhat underestimated from the standpoint of human malignant epithelium *per se.*[2]

Two of the analyses of human kidney cortex (Experiment 5, Table III) were upon fresh material obtained through heminephrectomy, performed because of the presence respectively of a bifurcated ureter and a pelvic stone.   The third analysis was upon a specimen secured 4 hours post mortem.   The respective C values in these three instances were 53, 35, and 47—all very low in contrast with the very high values for the same tissue in the rat.   It is highly probable that the mean value of 45 is representative of the normal, functioning kidney cortex of man.   This conclusion is fortified by the fact that these three kidneys were clinically and histologically normal.   A fourth non-functioning kidney, secured through nephrectomy, contained insufficient cytochrome c to be detected by our method of analysis.

By comparing the experimental results upon the seven species dealt with in Tables II and III the impression is gained of the probable existence of a hitherto unrecognized inverse relationship between the quantity of cytochrome c in a given type of epithelium and the body mass.[3]   The general trend of the C and (C/P) values is obviously in this direction, except for certain evident irregularities.   The latter consist of (1) somewhat lower values for the kidney cortex and liver of the mouse (Experiments 1 and 7, Table III) than for the corresponding rat tissues (Experiments 1 and 2, Table II), (2) an appreciably lower value for the kidney cortex of man than that of the horse or pig (Experiments 4 to 6, Table III), and (3) a somewhat

[2] The Walker carcinoma was derived originally from the mammary gland of the rat, and has at present the morphological structure of a carcinosarcoma, composed almost exclusively of neoplastic cells.   On the other hand, a quantitative microscopic survey of the six spontaneous neoplasms of man revealed their average cellular composition exclusive of migratory cells to be 56 per cent malignant epithelium, 22 per cent connective tissue, 12 per cent smooth muscle, 7 per cent normal mucosa, and 3 per cent lymphatic tissue and colloidal matter.   Most of the non-cancerous components appear to have lower C values than 30 γ per gm. of dry weight of tissue (Experiment 17, Column 6, Table III), found for the whole tumor mass.   For example, the smooth muscle from the stomach of man has been found by us to have a value of 22 γ, while, at least in the case of the rat, our analyses indicate that lymphatic and connective tissues have even lower values.

[3] In evaluation of the results with reference to body mass the following values may be employed for the average weight of individuals in our study, mouse 20 gm., rat 200 gm., rabbit 1700 gm., dog 8 kilos, pig 60 kilos, man 70 kilos, and horse 500 kilos.

higher value for rabbit lung (Experiment 15, Table III) than for the same tissue of the rat (Experiment 7, Table II).

It may be noted from the data in Table III that the decrease of the cytochrome c content with increase in body size of the species is far more pronounced in the kidney cortex series (Experiments 1 to 6) than in those types of epithelium (Experiments 13 to 17) which in the rat made up the low level cytochrome c group. This is seen most clearly by comparing the ratio of the cytochrome c content of the kidney cortex to that of colon mucosa in different species. These tissues lend themselves to such a comparison since, as has been mentioned, their P values (relative cellularity) are characteristic of the tissue and independent of the species of origin. The kidney cortex-colon mucosa ratios for rat, rabbit, and man are 11, 4, and 1 respectively on the basis of the C values, and 25, 7, and 2 on the basis of the (C/P) values. Similar ratios for the cytochrome c content of kidney cortex and tumors may be calculated for rat and man, yielding respective ratios of 20 and 2 (from the C values) and 52 and 3 from the (C/P) values. It is thus evident that the cytochrome c level of the kidney cortex, which is exceptionally high in the rat, is of "tumor magnitude" in man.

DISCUSSION

Since the cytochrome c content of most of the tissues studied by us has not been determined previously, only a few of the analytical values can be compared with those in the literature. The general order of magnitude of cytochrome c concentration which we find in the various epithelial tissues of the rat is qualitatively similar to that reported by Stotz (3) and Potter and DuBois (10). As we have already pointed out (6), our value for the kidney cortex of the rat agrees with that found by the above investigators. Our results upon rat liver and rat lung, on the other hand, differ significantly from those of the earlier workers, the value for liver being 3 times higher and that for lung only one-third as high. The exact reason for such discrepancies is problematical. For one thing, a common basis, such as the protein-bound phosphorus content, for the evaluation and comparison of results has not been available hitherto in this field.

When converted into our terms (C values) the cytochrome c content of nine types of neoplasms (carcinomas and sarcomas from the mouse, rat, and chicken), which were analyzed by DuBois and Potter (5), falls between 22 and 115. A C value of approximately 13 may be calculated for the adenocarcinoma of human liver analyzed by Junowicz-Kocholaty and Hogness (2). The range found by us for rat tumors, including three types of sarcomas not listed in Table II, is 21 to 136, and that for the adenocarcinomas of man (Table III) is 8.4 to 55. The data of different workers

upon the cytochrome c content of various types of neoplasms are, therefore, in good agreement.

The exceptionally low value found by us for the normal kidney cortex of man is none the less consonant with the order of magnitude of the cytochrome c content of the kidney of other large species. Our value for the kidney cortex of the horse is low, and of a magnitude similar to that which has been found for bovine kidney (2, 4).

The character of the cytochrome c-body weight correlation may provisionally be expressed in mathematical terms by plotting the logarithms of the C values (log C) against the logarithms of the average weights' (log W). When this is done for the C values of the kidney cortex series, the data may be fitted to two intersecting straight lines, represented respectively by the equations

$$\log C = 3.28 - 0.197 \log W \tag{1}$$

$$\log C = 4.49 - 0.592 \log W \tag{2}$$

Equation 1 represents the line connecting most of the values (those obtained from the mouse, rabbit, dog, pig, and horse), while Equation 2 is for the line based upon the results in rat, rabbit, and man.[4] A similar plot utilizing the data of the colon mucosa series yields a straight line practically parallel with that given by Equation 1, suggesting that this equation approximates the more common relationship of cytochrome c content and body mass. At present our data are too sparse to permit more than a limited conclusion that the cytochrome c content varies inversely with some fractional power of the body weight. The data upon the kidney cortex of rat and man are out of line with the correlation found for the cytochrome c content of this tissue in the other species. It is of interest to note that the rate of oxygen consumption of tissue slices has been stated (11) to be inversely proportional to $-0.24 \log W$, a value very similar to that found by us in Equation 1. The rôle of cytochrome c in cellular oxidation-reduction is well recognized. The use of the concentration of the pigment as a measure of the cytochrome oxidase system has been suggested (1), and will be further dealt with by us elsewhere.

Sincere thanks for furnishing material used in this study are due to the following, Dr. Shields Warren, Harvard Medical School, for two animals

[4] It is uncertain whether the deviation of the cytochrome c values of the kidney cortex of rat and man from the relationship expressed in Equation 1 may be ascribed to functional or anatomical peculiarities of these species. However, in this connection it may be mentioned that our analysis of the cytochrome c content of the external medulla of the human kidney yielded a value 70 per cent higher than that of the cortex. In the rabbit, dog, and pig, on the other hand, the values in medulla and cortex were either of the same magnitude or somewhat lower in the medulla.

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SUMMARY

The cytochrome c concentration has been determined in normal and neoplastic epithelial tissues of several species. The analytical results have been referred to the protein-bound phosphorus content of the tissues. The advantages of this type of reference have been discussed.

Significantly different values were obtained for the cytochrome c content of the same tissues from different species. This has led us to correlate the cytochrome c content with the factor of body mass. An inverse correlation of the cytochrome c content with body weight was found for the kidney cortex of mouse, rabbit, dog, pig, and horse. The values for the kidney cortex of rat and man did not fit the same mathematical relationship.

With reference to their content of cytochrome c the normal epithelial tissues of the rat fell into a high and low level group. Rat tumors were uniformly low in cytochrome c, but no lower than some of the normal epithelial tissues.

Contrasting values for the cytochrome c content of normal kidney cortex were obtained in the rat and man. An unusually high value was found in the former, an exceptionally low value in the latter species. Thus, the ratio of the cytochrome c content of kidney cortex to that of neoplasms was high in the rat, and practically of negligible magnitude in man, the species with the highest tendency towards spontaneous malignancy.

A deficiency of cytochrome c cannot be regarded as exclusively characteristic of malignant neoplasms.

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THE UTILIZATION OF LACTOSE BY THE FASTING WHITE RAT

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The comparative rates of absorption of the three naturally occurring monosaccharides, d-glucose, d-fructose, and d-galactose, from the intestine of the fasting white rat have been studied by the technique of Cori (1) in various laboratories. Although there has been considerable interest in the enzymè lactase, and studies of its activity *in vitro* have been made (Cajori (2)), there are comparatively few data available concerning the rate of hydrolysis of lactose, the sole carbohydrate of milk, in the gastrointestinal tract of the intact animal. In the present paper, a study of the hydrolysis of this sugar in the intestine of the albino rat is reported.

The extent to which glycogen is deposited in the liver of the fasting white rat following the administration of definite quantities of the common monosaccharides has also been the subject of numerous reports from various laboratories. Since there are few if any reliable figures in the literature relative to the content of liver glycogen following the administration of single doses of lactose to fasted rats, the present investigation on the fate of lactose in the intestinal tract was extended to include a study of the liver glycogen of such animals.

EXPERIMENTAL

The animal technique used in the present work was, with minor modifications, essentially that developed by Cori (1) for the study of the rate of absorption of sugars from the gastrointestinal tract of rats. Young rats which averaged 160 gm. in weight were fasted for 24 hours and were then given a 20 per cent solution of lactose by stomach catheter. Each rat received approximately 2 gm. of lactose per kilo of body weight. Higher concentrations of lactose were avoided because of the possibility of diarrhea. After a suitable period (1, 2, or 3 hours), the animal was killed by a sharp blow on the head and immediately decapitated to insure a rapid draining of blood from the tissues. The liver was removed as rapidly as possible, weighed, cut into small pieces, and quickly transferred to boiling alcoholic potassium hydroxide. Liver glycogen was then determined by the method of Good, Kramer, and Somogyi (3).

After ligation of the esophageal and rectal ends, the entire gastrointestinal tract was removed from the body and, after the extraneous fat was dis-

carded, was cut into small pieces. This material was repeatedly extracted with small portions of hot water, and the combined washings deproteinized with alkaline zinc salts as recommended by Somogyi (4). Portions of this filtrate were analyzed for glucose, galactose, and lactose, by the procedure suggested by Scott and West (5). It was necessary to make several modifications of this method in order to obtain consistent results. A technique was developed by which relatively small amounts of glucose and galactose (0.3 to 0.5 mg.) could be determined in the presence of greater quantities of lactose (1.0 to 2.0 mg.). Mixtures of the three pure sugars in water could be analyzed with an average error of less than 5 per cent for lactose and glucose and less than 10 per cent for galactose. For the application of this method to the determination of these sugars in the protein-free filtrates of intestinal contents, further modifications of the method were employed in order to obtain an accuracy comparable to that obtained in the analysis of pure sugar mixtures. These modifications were necessitated by the presence of certain unknown substances in the deproteinized intestinal filtrates. These substances in the intestinal filtrates of control fasted rats gave negligible reduction values but their reducing power was augmented in the presence of added sugars, especially after the acid hydrolysis which was necessary for the determination of lactose. A method was also developed for the analysis of glucose, galactose, and lactose in blood. To blood containing 110 to 130 mg. of glucose per 100 cc. were added 30 mg. each of galactose and lactose. The recoveries of the added galactose ranged from 90 to 100 per cent, as compared with recoveries of 80 to 100 per cent for the added lactose. The details of the above procedures are found in the doctoral thesis of Nalder (6) on file in the Library of the University of Michigan and available in microfilm.

Results

The results of the study are presented in Table I. With the exception of Rats 9 and 14, which weighed 133 and 129 gm., respectively, all of the remaining rats weighed from 149 to 180 gm. before the 24 hour fasting period. It is to be noted that in most of the experiments the hydrolysis and absorption were allowed to proceed for 3 hours before the animal was sacrificed. For comparison, a few experiments with 1 and 2 hour absorption periods were made. The amounts of galactose and lactose in the intestine at the end of the experimental period are recorded in Columns 5 and 6. Although the intestinal contents were analyzed for glucose as well as for galactose and lactose, these values are not recorded in the table, since in all of the experiments except two the amounts ranged from 0 to 6 mg. with an average value of 2.0 mg. of glucose per rat, regardless of the length of the experimental period (i.e., 1, 2, or 3 hours). Since such an

amount of glucose in the entire intestinal contents corresponds to 0.04 mg. of glucose in the aliquot analyzed, it was considered within the experimental error of the method and the conclusion was drawn that glucose was ab-

TABLE I

Hydrolysis of Lactose and Absorption of Its Hydrolytic Products

The lactose was administered in 20 per cent solution. The rats in Group II received, in addition to lactose, olive oil to provide 80 per cent of the weight of the lactose.

Group No.	Rat No. and sex	Lactose fed	Experimental period	Intestinal contents		Lactose hydrolyzed		Galactose absorbed		Liver glycogen
				Galactose	Lactose	Total	Per 100 gm. rat	Total	Per 100 gm. rat	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)
		mg.	hrs.	mg.	mg.	mg.	mg.	mg.	mg.	per cent
I	1 M.	362	3	13	98	264	152	127	73	1.40
	2 "	304	3	15	89	215	136	99	63	1.31
	3 "	272	3	11	75	197	128	93	61	1.20
	4 "	314	3	15	83	231	148	108	69	1.47
	5 "	340	3	12	93	247	152	119	73	0.99
	6 "	372	3	12	71	301	167	148	82	1.46
	7 "	366	· 3	11	80	286	160	141	79	1.62
	8 F.	282	3	20	30	252	161	113	72	1.11
	9 "	266	3	12	91	175	132	81	61	1.16
	10 "	344	3	15	86	258	156	122	74	0.93
	11 "	316	3	12	144	172	113	78	51	0.82
II	12 M.	250	3	12	38	212	145	100	68	1.15
	13 "	332	3	17	88	244	156	112	71	1.39
	14 F.	286	3	19	79	207	160	91	71	0.87
III	15 M.	374	2	21	150	224	124	98	55	1.27
	16 F.	318	2	15	124	194	124	88	56	1.01
	17 "	336	2	17	147	189	114	83	50	0.70
IV	18 M.	320	1	14	241	79	52	28	18	0.63
	19 F.	310	1	15	232	78	52	27	18	0.37
	20 "	312	1	13	168	144	97	63	42	0.47
V	21 M.*	346	3	33	100	246	146	98	58	0.64
	22 "*	368	3	28	155	213	118	85	47	0.87
	23 "*	338	2	16	252	86	52	30	18	0.56
	24 F.*	332	3	8	156	176	114	85	55	1.35
	25 "*	334	3	26	130	204	135	80	53	0.66

* Rats 21, 22, and 23 of Group V received calcium chloride and lactose (0.57 per cent calcium in 20 per cent lactose solution), while Rats 24 and 25 received, in addition to calcium chloride and lactose, an amount of olive oil equal to 80 per cent of the amount of lactose.

sorbed as rapidly as it was released by hydrolysis of the lactose. The two experiments in which a greater amount of glucose was found in the intestinal contents will be discussed later.

The total amount of galactose remaining in the intestinal contents of Rats 1 to 20 inclusive ranged from 11 to 21 mg., with an average of 15 mg. These values were essentially the same for the 1, 2, and 3 hour experimental periods. The figures indicate a slower rate of galactose absorption as compared with glucose when both sugars are present in the absorption mixture. This confirms the earlier work of Cori (7), who noted that, if a mixture of glucose and galactose were administered to white rats, the glucose was more rapidly absorbed than the galactose, whereas if the same sugars were administered singly, galactose was absorbed at a greater rate than glucose. The three experiments (Rats 21, 22, and 25) in which slightly higher levels of galactose were found in the gut will be discussed later.

The difference between the amount of lactose administered and that found in the gut at the end of the experimental period is recorded in Column 7 as the lactose hydrolyzed. This calculation is based on the assumption that none of the lactose is absorbed as such. Folin and Berglund (8) and Goldblatt (9) report that lactose is found in the urine of subjects receiving moderate amounts of this sugar by mouth. Definite proof that the sugar in the urine was lactose is not found in these papers. Cori and Cori (10), on the other hand, assume that the sugar excreted by rats after lactose feeding is galactose. Schantz, Elvehjem, and Hart (11) found a non-fermentable sugar in the urine of young rats which had been fed several weeks on a diet of skim milk, and definitely identified this sugar as galactose. This latter finding has been confirmed in this laboratory (Christman and Yeh[1]). It is probably of little importance for the present calculations whether the sugar is galactose or lactose, since at the level of lactose fed in the present experiments, one might predict from the work of Cori and Cori (10) that not more than 5 mg. of sugar would be excreted.

Rats in Group I (Rats 1 to 11 inclusive), receiving approximately 2 gm. of lactose per kilo of body weight, hydrolyzed from 54 to 89 per cent of this sugar during the 3 hour experimental period. Eight of the eleven values ranged from 71 to 81 per cent with an average for the entire group of 73 per cent. The average percentage hydrolysis of lactose for the 1 and 2 hour periods (Groups IV and III) was 32 and 59, respectively. The lactose hydrolyzed per 100 gm. of rat (Column 8) for rats in Group I varied from 113 to 167 mg., with an average value of 146 mg. The corresponding average values for the 1 and 2 hour periods (Groups IV and III) were 67 and 121 mg., respectively. From these average values it may be calculated that the amount of lactose hydrolyzed per 100 gm. of rat decreases from 67 mg. for the 1st hour to 54 mg. and 25 mg. for the 2nd and 3rd hours. The marked decrease in hydrolysis during the 3rd hour is not unexpected, since the concentration of the substrate is appreciably lower at the end of 2 hours.

[1] Christman, A. A., and Yeh, H. L., unpublished data.

The amount of galactose absorbed (Columns 9 and 10) is calculated by deducting the galactose found in the gut at the end of the experimental period (Column 5) from that which was formed by the hydrolysis of lactose (the figures in Column 7, multiplied by 0.53). Although the total galactose absorbed during a definite time interval varies considerably from rat to rat, a much better agreement is noted if a comparison is made of the galactose absorbed per 100 gm. of rat. The galactose absorbed per 100 gm. of rat for the 3 hour period (Group I) ranges from 51 to 82 mg. Eight of the eleven values fall between 61 and 74 mg., with an average of 69 for the entire group. The corresponding averages for the 1 and 2 hour experimental periods (Groups IV and III) are 26 and 54 mg., respectively. Therefore the galactose absorbed per 100 gm. of rat per hour for the 1st, 2nd, and 3rd hours would be 26, 28, and 15 mg., respectively. The only figures in the literature to which these values may be compared are found in the paper by Cori and Cori (10). On the basis of the discussion of the results presented in Table I of their paper, it may be calculated that the galactose absorbed per 100 gm. of rat per hour following the feeding of lactose was 30, 25, and 31 mg. for the 1st, 2nd, and 3rd hours. These values, however, were reported as average results of unpublished experiments, and no data are given concerning the amount of lactose fed, or the methods used in the analysis. The decrease in galactose absorption during the 3rd hour of the present experiments is similar to the decrease in the absorption of glucose reported by MacKay and Clark (12) to occur as the concentration of glucose in the intestine falls. It has been shown above that an average of 67, 54, and 25 mg. of lactose per 100 gm. of rat was hydrolyzed during the 1st, 2nd, and 3rd hours after feeding. Thus 36, 29, and 13 mg. of both glucose and galactose per 100 gm. of rat were available for absorption for these periods. Since the glucose which is formed by the hydrolysis of lactose is completely absorbed, 36, 29, and 13 mg. would represent the hourly absorption of the glucose derived from the lactose. Obviously the absorption rates of both glucose and galactose are a function of the rate of hydrolysis of the lactose. It would have been of interest to see whether a greater absorption of these sugars would have been obtained if the amount of lactose administered had been doubled or tripled. Fig. 1 in the paper of Cori and Cori (10) indicates the absorption of as much as 500 mg. of galactose after the feeding of lactose, but no statement is made as to the time required for such absorption nor of the amount of lactose administered. In the present work (see the later discussion) it was noted that after the administration of 3 and 4 gm. of lactose per kilo of rat the increases in blood sugar over normal values were not significantly greater than those produced by the feeding of 2 gm. of lactose per kilo.

Schantz, Elvehjem, and Hart (11) have reported that young rats fed on a mineralized skim milk diet for a period of several weeks have a high blood

sugar and excrete galactose in the urine. After the addition of fat to such a diet, the blood sugar returned to normal levels and the excretion of galactose stopped. These workers believe that fat is in some manner essential for the metabolism of galactose. Their work did not indicate whether the actual presence of fat in the gut was essential for the more efficient utilization of galactose. Therefore it was of interest to determine whether fat fed with lactose would alter the rate of hydrolysis of the sugar or the absorption of its hydrolysis products. Three rats (Group II) received such a volume of an olive oil-lactose emulsion as to provide approximately 2 gm. of lactose per kilo and an amount of olive oil which was approximately 80 per cent of the weight of the lactose. Such a mixture provides fat and lactose in approximately the same ratio as in milk. Since the percentages of lactose hydrolyzed and galactose absorbed were essentially the same for these rats as for the rats receiving lactose alone (Group I), it may be concluded that the presence of the fat did not inhibit the hydrolysis of lactose or the absorption of glucose and galactose.

Since Gardner and Burget (13) have noted that calcium salts depressed the absorption of glucose from the intestine of dogs and rats, a study of the effect of calcium ions on the hydrolysis of lactose and the subsequent absorption of glucose and galactose was made. Three rats (Nos. 21, 22, and 23 of Group V) received a lactose-calcium chloride mixture and two rats (Nos. 24 and 25 of Group V) received lactose, olive oil, and calcium chloride. As in the previous experiment the amount of oil administered was roughly four-fifths of the lactose. The calcium content of the solutions which were fed was adjusted to provide a lactose-calcium ratio similar to that found in whole milk, approximately 35:1. The average amount of lactose hydrolyzed per 100 gm. of rat for a 3 hour experimental period (Rats 21, 22, 24, and 25) was 128 mg. as compared with a value of 146 mg. for the rats of Group I, which received a pure lactose solution. The amount of lactose hydrolyzed per 100 gm. of rat during a 2 hour experimental period (Rat 23) is only 52 mg. as compared with an average of 121 mg. per 100 gm. of rat for the rats of Group III, receiving pure lactose solution.

The amount of galactose remaining in the intestine of Rats 21, 22, and 25 at the end of the 3 hour period is also definitely greater than that found in the intestine of any of the rats in Groups I to IV, inclusive. It is also notable that in the case of Rats 21 and 25 appreciable amounts of glucose (24 and 22 mg., respectively) are present in the intestine at the end of the 3 hour period, whereas only negligible amounts of glucose were found in the gut of the twenty rats in the first four groups.

Because of the slower rate of lactose hydrolysis and decreased rate of galactose absorption in rats receiving calcium chloride, the amount of galactose absorbed per 100 gm. of rat during a 3 hour experimental period

averaged 53 mg. as compared with 69 mg. for rats of Group I. Only 18 mg. of galactose per 100 gm. of rat were absorbed during a 2 hour experimental period (Rat 23), as compared with an average value of 54 mg. for the rats of Group III. Although the number of rats used in these latter experiments is small, the results seem to indicate that the presence of calcium salts with the lactose in the intestine decreases the rate of lactose hydrolysis as well as of galactose absorption. The olive oil was included in the lactose-calcium salt mixture fed to Rats 24 and 25, to determine whether the presence of fatty acids resulting from the hydrolysis of the fat would modify the effect of the calcium. Since the amount of lactose hydrolyzed and the galactose absorbed for Rats 24 and 25 is essentially the same as for Rats 21 and 22 which received a calcium chloride-lactose mixture, it may be concluded that the effect of the calcium salts is not altered by the presence of fat. Cori and Cori (10) have pointed out that the absorption of galactose resulting from the hydrolysis of lactose fed in the form of milk proceeds at a slower rate than from lactose fed in pure aqueous solution. The slower rate of absorption, attributed by these workers to the simultaneous absorption of amino acids, may have been due to the presence of calcium ions.

Liver Glycogen

As previously pointed out, few determinations have been reported of the liver glycogen content of rats which had received a single dose of lactose by stomach tube following a 24 hour fasting period. The liver glycogen of the twenty-five rats used in the study of lactose hydrolysis is reported in Table I. The liver glycogen of additional rats which had been fed lactose with or without other supplements has also been determined. Since complete absorption studies were not made on these rats, these experiments are not recorded in Table I.

An examination of Table I indicates that the average glycogen content of the livers of rats killed 3 hours after the administration of lactose is somewhat over 1 per cent. It is to be noted that the liver glycogen of the male rats is in most cases distinctly higher than that of the female rats. Such a sex variation in the liver glycogen of male and female rats has been previously reported by Deuel and coworkers (14) and Blatherwick *et al.* (15). The liver glycogen of nine male rats (two not included in Table I) for the 3 hour experimental period ranged from 0.99 to 1.62 per cent, with an average value of 1.34 per cent. The corresponding values for four female rats varied from 0.82 to 1.16 per cent, with an average value of 1.01 per cent. The single female in Group II has a liver glycogen content of 0.87 per cent, as compared with an average value of 1.32 per cent for the three males (one not included in Table I). The same sex variation in liver

glycogen is to be observed in the male and female rats of Groups III and IV.

As previously shown, the lactose hydrolyzed per 100 gm. of rat during the 3, 2, and 1 hour periods averaged 146, 121, and 67 mg., respectively. Since the glucose made available by this hydrolysis is completely absorbed, the corresponding amounts of glucose absorbed per 100 gm. of rat would be 77, 64, and 36 mg., respectively. Since the average galactose absorbed per 100 gm. of rat for the corresponding periods was shown to be 69, 54, and 26 mg., respectively, the total sugar absorbed for the 3, 2, and 1 hour periods would be 146, 118, and 62 mg. The corresponding ratios for these absorption values are 2.4, 1.9, and 1.0. The average liver glycogen, expressed in per cent of liver weight, for these rats (both male and female) was 1.22, 0.99, and 0.49, ratios of 2.5, 2.0, and 1.0, for the 3, 2, and 1 hour periods. It is thus seen that the ratios for the liver glycogen and the total amount of sugar absorbed are roughly proportional. If the average total glucose and galactose absorbed in these periods regardless of the weight of the animal (237, 197, and 90 mg.) are compared to the average liver glycogen expressed in mg. (71, 61, and 31), it is apparent that the amount of glycogen deposited in the liver represents approximately one-third of that which could have been formed from the total amount of absorbed sugar.

In general, the values given in the literature for the liver glycogen of rats following the administration of glucose cannot be compared with the values obtained after lactose administration, since the amounts of glucose employed have usually been much larger than 2 gm. per kilo. In the present experiments, the dosage was of necessity small because of the relative insolubility of lactose compared with that of glucose, and the tendency to diarrhea caused by large amounts of lactose in the gut. In their experiments on xylose metabolism, Miller and Lewis (16) fed rats ranging in weight from 163 to 179 gm. amounts of glucose varying from 226 to 266 mg. These small amounts of glucose were fed in order that the amounts absorbed would be comparable to the amount of xylose absorbed in the same period. An average value of 47 mg. of glucose was absorbed per 100 gm. of rat per hour. In the present work, rats which weighed 25 to 30 gm. less than those used by Miller and Lewis received approximately 300 mg. of lactose: The average absorption of glucose plus galactose per 100 gm. of rat per hour for the 3 hour period was 49 mg. (calculated from the averages given above). The average liver glycogen after glucose administration reported by Miller and Lewis was 0.63 per cent for a 3 hour period, as compared with an average value of 1.22 per cent in the present experiments.

Cori (17) has pointed out that galactose in comparison with glucose and

fructose is a poor glycogen former. This was in part explained by the large proportion of absorbed galactose which was excreted in the urine. In a subsequent paper, Cori and Cori (18) stated that a higher percentage of the absorbed galactose was lost by urinary excretion than was indicated in the earlier work. After suitable corrections have been applied for the urinary loss of galactose, it may be calculated from Table IV of Cori's paper (17) that with an average of 79 mg. of galactose available per 100 gm. of rat per hour, a corrected liver glycogen value of 0.68 per cent was obtained at the end of a 3 hour experimental period. By comparison, in the present work an average absorption of 49 mg. per hour per 100 gm. of rat of glucose and galactose derived from the hydrolysis of lactose produced, in 3 hours, a liver glycogen of 1.16 per cent. This average value includes the liver glycogen from both male and female rats. If the liver glycogen values of the male rats are averaged, the value of 1.28 per cent is obtained. This probably is a fairer method of comparison, since only male rats were used in the Cori study. It would appear then that the inefficiency of galactose as a glycogen former is not entirely explained by its rapid urinary excretion following absorption. The better utilization of galactose from the hydrolysis of lactose is probably explained by the slower rate of absorption due to the slow hydrolysis of lactose and the simultaneous absorption of glucose.

The liver glycogen content of rats in Group II which received olive oil with lactose is approximately the same as for those in Group I which received only lactose. The lower liver glycogen of male Rats 21, 22, and 23, which received lactose with calcium chloride, is consistent with the finding of increased amounts of unabsorbed galactose and glucose in the intestinal tract. The galactose absorption per 100 gm. of rat per hour for these rats is much lower than for the male rats which received comparable amounts of lactose without the calcium salts. Although the galactose absorption of the female rats (Nos. 24 and 25) which received lactose olive oil, and calcium is somewhat below the average noted for rats in Groups I and II, the liver glycogen for Rat 24 is higher than that for any of the female rats of these groups. On the other hand, Rat 25 has a liver glycogen which is somewhat lower than that of the females in Group I or II. An unabsorbed residue of 22 mg. of glucose and 26 mg. of galactose was found in the intestinal tract of Rat 25 at the end of the 3 hour absorption period.

Blood Sugar

In connection with the absorption studies, a limited number of blood sugar determinations were made. Since there are few figures in the literature relative to the increase in blood glucose of rats following the oral administration of glucose, a preliminary study with this sugar was made.

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REMOVAL OF CANAVANINE FROM PREPARATIONS OF JACK BEAN UREASE

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The presence of canavanine in jack beans has been demonstrated by Kitagawa (1). Both Kitagawa (2) and Damodaran (3) have shown that liver arginase reacts with canavanine, yielding urea and canaline, but that the arginase liberates urea much more slowly from canavanine than from arginine.

Hunter and Pettigrew (4) have shown that if urease and arginase are allowed to react simultaneously in the determination of arginine it is necessary to use a "ripened" mixture of the two enzymes; *i.e.*, one which has already stood for 24 hours at room temperature. Otherwise yields of CO_2 indicate arginine values 0.7 per cent lower than those obtained when the urease acted for only a few minutes. No explanation for this phenomenon was offered. However, it now seems likely that with the unripened mixture, through action of arginase on the canavanine in the urease preparation, more CO_2 was produced in the blank than was produced from this source in the unknowns which contained other substrate (arginine) and inhibitors (such as ornithine (5) or, in the case of protein hydrolysates, amino acids other than arginine (6)). Consequently it is desirable to have a canavanine-free urease for use in the arginase method of determining arginine.

A urease preparation low in free amino acid is desirable also in removing urea from biological material before quantitative determination of free amino acids by the ninhydrin-CO_2 method (7, 8).

We find that urease prepared from jack beans by the commonly used acetone precipitation method of Van Slyke and Cullen (9) contains about 6 mg. of α-amino nitrogen per gm. of crude dry urease, corresponding to a canavanine content of about 80 mg. per gm.

The following procedure removes almost completely canavanine and what other amino acids may be present.

Procedure

A uniform paste is made with 20 gm. of Squibb's[1] Double Strength urease and water and is made up to 50 cc. with water. Centrifugation at 2000 R.P.M. for 2 minutes removes 0.38 gm. of insoluble material. The super-

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[1] This urease is prepared by the acetone precipitation method of Van Slyke and Cullen (9).

natant is dialyzed at 0° in NoJax cellulose[2] sausage casing against 2 liters of distilled water (with rocking, and with a marble in the sac for stirring) for 6 to 8 hours. Six subsequent dialyses of 6 to 8 hours each against 2 liters of 0.001 M phosphate of pH 6.5 reduce the amino acid content to minimal values. The urease solution is then frozen and thoroughly dried in a vacuum while frozen. The resulting powder is readily soluble in water and its urease activity per mg. is of the same order as that of the starting material. Approximately 50 per cent of the weight of Double Strength urease passes through the membrane. Analyses of two typical preparations are summarized in Table I.

That nearly all of the carboxyl nitrogen is derived from canavanine is indicated by analysis of the dialysate. 18 gm. of Double Strength Squibb's

TABLE I

Effect of Dialysis on Activity and α-Nitrogen Content of Acetone-Precipitated Urease

Preparation No.	Urease activity		Weight recovered	Carboxyl nitrogen present[†]		Carboxyl nitrogen left after dialysis
	Before dialysis	After dialysis		Before dialysis	After dialysis	
	Sumner units[*] per mg.	*Sumner units[*] per mg.*	*per cent*	*mg. COOH-N per gm.*	*mg. COOH-N per gm.*	*per cent of original*
1	0.41	0.477	49.6	5.97	0.214	3.6
2	0.361	0.277	49.1	5.92	0.157	2.6

[*] 1 unit of urease (10) is taken as the amount forming ammonia at the rate of 1 mg. of ammonia N per 5 minutes when the temperature is 20° and urea concentration 3 to 6 per cent and the concentration of phosphate (pH 6.8) is 0.05 M.

[†] "Carboxyl nitrogen," according to the usage of Van Slyke, Dillon, MacFadyen, and Hamilton (11), indicates α-amino nitrogen calculated from the CO_2 yielded from the carboxyl groups of $\cdot CH(NH_2) \cdot COOH$ and $\cdot CH(NH) \cdot COOH$ groups in the ninhydrin-CO_2 method of analysis for free amino acids.

urease were dissolved in 50 cc. of H_2O and dialyzed against 50 cc. of water for 6 hours. The protein-free solution outside the sac was analyzed. The following results (Table II) are consistent with the probability that the nitrogen was practically all in canavanine. (a) The α-nitrogen was 26.7 per cent of the total. The structures of arginine and canavanine, *viz.* $NH_2 \cdot C(NH) \cdot NH \cdot (CH_2)_3 \cdot CH(NH_2) \cdot COOH$ and $NH_2 \cdot C(NH) \cdot NH \cdot O \cdot (CH_2)_2 \cdot CH(NH_2) \cdot COOH$, indicate that analysis by the ninhydrin-CO_2 method should yield CO_2 from each indicating 25 per cent of the total nitrogen in the α-amino group. (b) Under the conditions used for the hypobromite reaction we have found arginine to evolve 49 per cent of its nitrogen. We did not have pure canavanine to submit to the hypobromite reaction, but from the similarity of its structure to that of arginine a similar

yield would be expected from canavanine.   The results in Table II show 53 per cent.

These relations taken with the behavior of the dialyzed material towards arginase are consistent with the interpretation that practically all the nitrogen in the dialysate was in the form of canavanine.

Canavanine, arginine, and glycocyamine would be expected to give high ratios of "hypobromite N" to total N, but glycocyamine yields no CO_2 by reaction with ninhydrin, hence cannot contribute to the α-nitrogen found. The presence of appreciable concentrations of arginine or glycocyamine was ruled out by applying the quantitative Sakaguchi test after permutit fractionation according to the technique of Dubnoff (12).   The color obtained indicated that there could be present not more than 0.1 per cent of glycocyamine and not more than 0.4 per cent of arginine in the original urease.

The reaction rate of liver arginase on the material in the dialysate was followed over an 8 hour period by determining at intervals the amounts of urea set free in aliquot portions, which were analyzed by Van Slyke's

TABLE II

Analysis of Dialysate of Acetone-Precipitated Urease

	Nitrogen measured per gm. original urease	Proportion of total dialysate nitrogen
	mg.	*per cent*
α-N by ninhydrin-CO_2............	6.38	26.7
Total N (micro-Kjeldahl)................. .	23.97	100.0
N_2 liberated by hypobromite...............	12.73	53.2

manometric urease method (13), with urease purified as described in this paper, and the technique devised for blood urea analyses (13).   The amount of arginase present sufficed to decompose 99 per cent of arginine in 20 minutes.   In contrast, the urea formation in the dialysate was equivalent after 2 hours to 44 per cent, and after 7.5 hours to 75 per cent, of the canavanine estimated by total N.   This relatively slow rate of reaction with arginase is characteristic of canavanine.

A procedure for the quantitative determination of canavanine involving the specific nitroprusside reaction used qualitatively by Kitagawa is to be outlined in a subsequent paper.   This procedure was applied to the dialysates and indicated that the canavanine corresponded to the total nitrogen determined by the Kjeldahl analysis.

SUMMARY

1. Urease prepared from jack beans by the Van Slyke-Cullen method of acetone precipitation was found to contain 7 to 8 per cent of canavanine.

2. A method of purifying the urease by dialysis is described which removes more than 96 per cent of the canavanine. The purified urease is suitable for use in estimating the urea formed in Hunter's arginase determination of arginine and in freeing biological fluids of urea as a preliminary to amino acid determination.

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A DIRECT METHOD FOR THE DETERMINATION OF N-METHYL DERIVATIVES OF NICOTINIC ACID IN URINE*

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Studies on the metabolism of nicotinic acid have shown the importance of trigonelline as a normal excretory product (1–4) and, after a dose of nicotinamide, as an indication of nicotinic acid nutrition (5, 6). The recent work of Huff and Perlzweig (7) indicates that F_2, the fluorescent metabolite of nicotinic acid described by Najjar and Wood (8), may be identical with N-methylnicotinamide. This is closely related to trigonelline and may comprise a large part of what has been measured as trigonelline in earlier work. In the present communication a direct method for the determination of both trigonelline and N-methylnicotinamide in urine is presented.

The only useful methods which have been available for the determination of trigonelline are based on two types of reaction. The first of these is the conversion of trigonelline to nicotinic acid by hydrolysis with strong alkali in the presence of ammonia (or urea), followed by reaction with CNBr and an amine (2, 9, 10). The conditions proposed by Perlzweig, Levy, and Sarett (2), such as preliminary strong acid hydrolysis and addition of urea to the alkaline reaction, resulted in 65 to 75 per cent conversion. The omission of these two steps by Melnick, Robinson, and Field (10) resulted in a conversion of only 28 to 38 per cent. Trigonelline measurements by this type of method (2) are lengthy and require that nicotinic acid also be measured and subtracted. In the presence of comparatively large amounts of nicotinic acid the accuracy of the trigonelline determination is impaired.

The second type of reaction, proposed by Kodicek and Wang (11), is based on an alcoholic alkaline hydrolysis, followed by condensation with an amine, and is better suited for direct analyses for trigonelline. However, the method of Kodicek and Wang (11), as described, gave inconsistent results and trigonelline was lost in the charcoal clarification. The modification by Fox, McNeil, and Field (12) still leaves much to be desired, since the K value for trigonelline added to urine "varies for different urines and from the values in aqueous solution." The proposed method, also based on the Kodicek and Wang reaction (11), gives reproducible values

for total trigonelline-like compounds in urine and good recoveries of added trigonelline and N-methylnicotinamide.

Reagents—

11 N NaOH solution.

$Pb(NO_3)_2$ solution, 25 gm. dissolved in 95 ml. of water.

K_3PO_4, powdered.

95 per cent ethyl alcohol.

Concentrated HCl.

Phenolphthalein solution, 0.5 per cent in 50 per cent alcohol.

5 N HCl solution.

0.5 N NaOH solution.

Benzidine solution.   1 gm. of benzidine (Merck) is dissolved in 25 ml. of 95 per cent ethyl alcohol and 75 ml. of 0.75 N HCl solution.   This solution is kept in the refrigerator and is stable for at least 2 weeks.

Procedure

In an ordinary Pyrex test-tube (18 × 150 mm.) calibrated at 10 ml. is placed 1 to 5 ml. of urine which is then diluted with water to a volume of about 9 ml.   The best results are obtained when 50 to 150 γ of trigonelline are used as starting material, although 25 to 250 γ can be measured accurately by this method.   To the diluted urine 0.3 ml. of 11 N NaOH is added and the mixture is heated in a boiling water bath for half an hour. After cooling, the volume is adjusted to the 10 ml. mark and 3.3 ml. of $Pb(NO_3)_2$ solution are added to each tube.   The contents are well shaken by inverting the tube.   A drop of phenolphthalein is added, and, if the solution is still alkaline, a pinch of powdered $Pb(NO_3)_2$ is added, the solution is mixed again, centrifuged, and decanted into another test-tube.   The excess of lead is removed by the addition of a pinch of K_3PO_4 to alkalinity and centrifugation of the precipitate after 5 to 10 minutes.

In a 22 × 175 mm. Pyrex test-tube graduated at 30 ml. are placed 4 ml. of this solution, 16 ml. of 95 per cent alcohol, and 4 ml. of 11 N NaOH solution.   The contents are well mixed by inverting the tube, after which the tube is covered with a blown glass bulb and heated in a water bath at 75–80° for 45 minutes.   After cooling, 3.7 ml. of concentrated HCl are added and the contents again cooled and mixed by inverting the tube. After the addition of another drop of phenolphthalein, the solution is carefully adjusted by the dropwise addition of 5 N HCl solution to be just acid. With 0.5 N NaOH the mixture is made *just alkaline* to phenolphthalein. Water is added to the 30 ml. mark, and the solution is mixed and centrifuged to clear it of slight turbidity.

10 ml. of this solution (representing one-tenth of the original sample) are placed in an Evelyn colorimeter tube, 1 ml. of the benzidine solution is

added, and the solution is mixed by agitation. The color is read in 25 to 30 minutes in the usual way in the Evelyn photoelectric colorimeter with Filter 490. The color reaches a maximum in 20 minutes and remains stable for an additional 20 minutes, after which it fades very slowly. The reagent blank which consists of 4 ml. of water taken through the alcoholic alkaline hydrolysis and treated in the same manner is used to set the galvanometer at 100.

The treatment described above effectively decolorizes the urine so that controls are needed only when large amounts of urine of high specific gravity are taken for analysis. In these cases, another 10 ml. of the alcoholic alkaline digest are taken and 1 ml. of the alcoholic hydrochloric acid solution (used above for dissolving the benzidine) is added. This is read against the water blank treated in the same way and the value subtracted.

A set of sixteen analyses, including a standard and a blank, can be conveniently run in 5 to 6 hours.

Discussion of Method

The preliminary hydrolysis of the urine in 0.35 N alkali is necessary to convert N-methylnicotinamide to trigonelline before the alcoholic alkaline treatment. Without this first hydrolysis, N-methylnicotinamide yields only 40 to 60 per cent of the color obtained with an equivalent amount of trigonelline, and urine values are also lower. Since trigonelline is destroyed by strong alkaline hydrolysis, this point was checked for weak alkali and no destruction of trigonelline was detectable after hydrolysis with 0.3 to 0.7 N NaOH for 30 minutes in a boiling water bath. The use of lead nitrate for the clearing of the urines removes no trigonelline.

Fox et al. (12) have used methyl alcohol instead of ethyl alcohol to yield a monophasic reaction medium. The conditions as set forth here with the use of ethyl alcohol also result in a one phase reaction mixture and yield more color per mole of trigonelline than with methyl alcohol. Under these conditions benzidine also gives more color than the dianisidine employed by Fox et al. (12).

Since hydrolysis of trigonelline with strong alkali in the presence of urea yields nicotinic acid (2, 9) and since the amount of urea present varies for each urine sample, the effect of varying amounts of urea on the alcoholic alkaline hydrolysis was tested. There was no effect of urea in amounts up to 80 to 100 mg. in the original sample. In the presence of 150 mg. of urea the trigonelline values were about 15 per cent low. On this basis there is usually no interference in amounts up to 5 ml. of urine except in concentrated samples, but if this amount or more need be taken it is suggested that recovery of added trigonelline be tested at the same time.

Fox et al. (12) have also found that glucose interferes with their method

by increasing the color value. In the method given here 60 mg. of glucose may be present before the trigonelline value is increased. Increases of 1 to 2 γ are obtained in the presence of 80 to 100 mg. of glucose.

Pyridine compounds which have no methyl group on the ring nitrogen do not give any color by this reaction. N-Methylpyridinium hydroxide yields only about 5 per cent as much color as the equivalent amount of trigonelline. Nicotine yields no color.

The values obtained for urine and for standard solutions of trigonelline and N-methylnicotinamide are reproducible. The coefficient of variation

TABLE I

Recovery of Trigonelline and N-Methylnicotinamide Added to Urine

Urine No.	Urine	Added trigonelline*	Added N-methyl-nicotina-mide †	L values‡		Recovered	
				Urine	Urine and added substance		
	ml.	γ	γ			γ	per cent
		8			0.173	8	
			7.2		0.157	7.2	
1	0.3	4		0.022	0.115	4.3	107
1	0.3		3.6	0.022	0.097	3.5	97
2	0.1	4		0.461	0.549	4	100
2	0.1		3.6	0.461	0.538	3.5	97
3	0.1	4		0.067	0.149	3.8	95
3	0.1		3.6	0.067	0.141	3.4	95
4	0.4	4		0.179	0.258	3.7	93
4	0.4		3.6	0.179	0.258	3.6	100
5	0.4	4		0.113	0.195	3.8	95
5	0.4		3.6	0.113	0.197	3.8	106

* Added as trigonelline acid sulfate (9).

† Added as N-methylnicotinamide chloride (7).

‡ The L value is the optical density obtained from the galvanometer reading (G) by the formula, $L = 2 - \log G$.

for eleven standard trigonelline solutions run in seven separate experiments was 4.1 per cent. The color obtained obeys Beer's law and for solutions containing 2.5, 5, 10, 15, and 20 γ of trigonelline, the L values per microgram were 0.0211, 0.0210, 0.0220, 0.0217, and 0.0216, respectively. With urine the color obeys Beer's law until limited by an excess of urea. Table I shows the satisfactory recovery of trigonelline and N-methylnicotinamide which were added to urine containing varying amounts of trigonelline compounds.

In many urines trigonelline has been determined by this method and by

the procedure of Perlzweig, Levy, and Sarett (2). For most of the urines there was excellent agreement of results. Occasionally the present method inexplicably gave figures 20 to 30 per cent higher than those obtained by the old one. Table II shows some of these comparative values, and the F_2 figures which have been determined by Huff and Perlzweig[1] in many of these urines with N-methylnicotinamide as a working standard. The trigonelline values given for urine include N-methylnicotinamide and other methylated nicotinic acid derivatives which may be present. In control urines little F_2 was found compared to the total trigonelline present, but after a dose of nicotinamide most of the so called trigonelline is accounted for by the F_2 value. The differences between F_2 and total trigonelline

TABLE II

Trigonelline and F_2 Content of Control Urines and Urines after 500 Mg. Dose of Nicotinamide

Subject	Urine	Trigonelline by conversion to nicotinic acid (cf. (2))	Trigonelline by present method	F_2* by fluorescence
		mg.	mg.	mg.
A	Control (14 hrs.)	1.8	1.6	0.0
B		2.4	2.4	1.1
C		8.0	9.6	0.6
D		12.1	11.5	3.2
A	After 500 mg.	34	48	38
B	nicotinamide (14	83	86	67
C	hrs.)	87	83	77
"		86	88	62
D		110	112	119

* The values for F_2, given in terms of N-methylnicotinamide, were obtained by and are reproduced with the permission of Dr. W. A. Perlzweig (see foot-note 1).

values may be due in part to some trigonelline of dietary origin. A sample of F_2 isolated from urine by Huff and Perlzweig (7) gave the same amount of color by the present method as did an equivalent amount of N-methylnicotinamide.

Since both trigonelline and N-methylnicotinamide are measured by the method presented here, the use of this method and fluorescence analyses (F_2) can serve to partition these compounds in biological fluids. This procedure is being used to follow methylation *in vitro* of nicotinic acid compounds by tissue slices of different animals. Further data on this and on urine will be published later.

[1] Huff, J. W., and Perlzweig, W. A., unpublished results.

SUMMARY

A direct method for the determination of trigonelline and N-methyl-nicotinamide in urine is presented, based on an alcoholic alkaline hydrolysis and condensation with benzidine. The inherent difficulties and interfering substances are discussed.

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THE EQUILIBRIUM RELATIONS OF *d*-AMINO ACID OXIDASE, FLAVIN ADENINE DINUCLEOTIDE, AND AMINO ACIDS FROM KINETIC DATA*

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The kinetics of the reaction of substrates when catalyzed by enzymes have been formulated in a variety of ways. Michaelis and Menten (3) postulated a mass action equilibrium involving the substrate, the free enzyme, and an enzyme-substrate complex. When the reaction velocity was assumed to be proportional to the concentration of the enzyme-substrate complex, complex kinetic data in many cases were formulated with remarkable fidelity by the conception. The method is now widely used and the mass action constant derived from kinetic data is familiarly designated as the Michaelis-Menten constant. Lineweaver and Burk (2) treated the subject similarly and modified the mathematical presentation to permit easier presentation of experimental data and calculation of the constant.

Michaelis and Menten treated the case which is not dependent upon a coenzyme. The case of a protein enzyme system which is dependent upon the presence of a coenzyme for its catalytic action is an interesting variant. Such a system is *d*-amino acid oxidase first described by Krebs (1). Warburg and Christian (5) showed that the active enzyme is a protein-flavin adenine dinucleotide complex which catalyzes the oxidation of many *d*-amino acids to keto acids according to the equation (in the case of *d*(−)-alanine, for example) $CH_3 \cdot CHNH_2COOH + \frac{1}{2}O_2 = CH_3 \cdot CO \cdot COOH + NH_3$.

In the course of experiments on the possible action of insulin upon *d*-amino acid oxidase it became necessary to formulate the behavior of the oxidase system under two conditions; *viz.*, (1) when the coenzyme concentration is insufficient to saturate the enzyme completely, pH constant; (2) when the coenzyme is above saturation level and the pH is varied. In this paper we present theoretical and experimental studies in these two cases.

Methods

The coenzyme-free enzyme was prepared from sheep kidneys, according to the method of Negelein and Brömel (4). No attempt was made to carry the preparation to a high state of purity, and so the later steps of the method were omitted. However, freedom from coenzyme was obtained as shown by the complete inactivity of the preparation in the absence of added flavin adenine dinucleotide. The preparation was kept in the dry state, and for use was dissolved in the appropriate buffer.

The flavin adenine dinucleotide[1] was prepared by the method of Warburg and Christian (5). Since preparation of high purity FAD is attended with large losses, we omitted the later steps of the method. However, our preparations ranged from 0.05 to 0.20 in purity. For use the required amounts were weighed by a micro balance and dissolved in appropriate buffer.

The substrate in all cases was Hoffmann-La Roche *dl*-alanine. Since the enzyme system has no action upon $l(+)$-alanine, all concentrations of substrate are expressed in terms of $d(-)$-alanine.

Determination of Reaction Velocity—Required amounts of $d(-)$-alanine and FAD dissolved in buffer at the designated pH were placed in the main compartment of a Warburg respirator. The side sac contained the oxidase preparation dissolved in buffer. The vessels were equilibrated (air as the gaseous phase) at 38° until temperature equilibrium had been reached. The taps were then closed, the enzyme from the side sac quickly and thoroughly mixed with the contents of the main compartment, and the shaking resumed. Readings of the manometer were made at 5, 10, 15, and 20 minutes. These readings plotted against time usually gave a straight line from which the rate of oxygen uptake in micromoles per minute per mg. of the oxidase preparation was calculated.

Calculation of Molecular Concentration of Total FAD—Since the preparations of FAD used in these experiments were not carried through the final steps in the purification as outlined by Warburg and Christian (5), they were impure. However, it was possible to assay them accurately by making use of the data of Warburg and Christian who used preparations of FAD of purity 1.0. They found at pH 8.3 (pyrophosphate buffer) and 38° in the case of any preparation of amino acid oxidase in the presence of 0.021 M $d(-)$-alanine that the velocity of the reaction is a maximum at high concentrations of FAD but is half the maximum when the concentration of FAD is 0.196 γ per cc. or 0.25×10^{-6} mole per liter. Hence to assay a preparation of FAD, the velocity (V) of uptake of oxygen is determined under the above conditions with varying amounts of the flavin

[1] For brevity flavin adenine dinucleotide will be referred to as FAD.

preparation. The observed values of $1/V$ are plotted against the reciprocal of the concentrations of the FAD preparation. The straight line obtained gives by extrapolation at $1/(F) = 0$ the minimum value of $1/V$ (i.e., maximum V). At that point where $1/V$ is twice the minimum (i.e., $V = \frac{1}{2}V_{maximum}$) the true concentration of the FAD is 0.25×10^{-6} mole per liter. Comparison with the concentration of impure preparation at that point allows of the calculation of its purity. *All concentrations of FAD given in this paper are in terms of pure substance calculated from the concentration of impure preparation and the determined purity of the FAD preparation.*

Case I. Oxidase Incompletely Saturated with FAD or Amino Acid, pH Constant, and $t = 38°$

Symbols

V = velocity of the reaction in micromole of O_2 per minute per mg. of (impure) oxidase preparation

(P) = concentration (mole per liter) of total enzyme

(p) = "   "   "   "   " protein not combined either with FAD or amino acid

(PF) = concentration (mole per liter) of protein-FAD

(PFS) = "   "   "   "   " protein-FAD-amino acid complex

(S) = "   "   "   "   " $d(-)$-amino acid

(F) = "   "   "   "   " total FAD

(f) = "   "   "   "   " free FAD

K_{PFS} = dissociation constant of protein-FAD-amino acid complex

K_{PP} = "   "   " protein-FAD

k_{PFS} = a proportionality constant

In a system of oxidase, d-amino acid, and FAD, it may be assumed that the following dissociation reactions occur.

$$PF = P + F \tag{1}$$
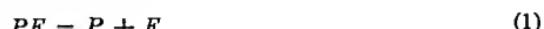
$$PFS = PF + S \tag{2}$$

Accordingly there may be written the following mass action equations.

$$K_{PF} = \frac{(p) \cdot (f)}{(PF)} \tag{3}$$

$$K_{PFS} = \frac{(PF) \cdot (S)}{(PFS)} \tag{4}$$

Furthermore there are also two stoichiometrical equations,

$$(P) = (p) + (PF) + (PFS) \tag{5}$$

$$(F) = (f) + (PF) + (PFS) \tag{6}$$

By combination of Equations 3, 4, and 5 the following expression is derived.

$$(P) = \left[\left(1 + \frac{K_{PFS}}{(S)}\right) + \frac{K_{PFS}K_{PF}}{(S)\cdot(f)}\right](PFS) \qquad (7)$$

But it is possible to assume that the velocity of the reaction of the amino acid oxidase system (expressed in some convenient terms such as micromoles of oxygen per minute per mg. of oxidase preparation) is proportional to the concentration of the active component PFS; that is, $V = k_{PFS}(PFS)$. Equation 7 then becomes

$$\frac{1}{V} = \frac{1}{k_{PFS}(P)}\left[\left(1 + \frac{K_{PFS}}{(S)}\right) + \frac{K_{PFS}K_{PF}}{(S)(f)}\right] \qquad (8)$$

Experimentally this equation is best investigated by maintaining (S) constant and allowing (f) to vary. For ease of reference the equation is simplified, (S) being constant, to the form

$$\frac{1}{V} = a_1 + \frac{b_1}{(f)} \qquad (9)$$

where

$$a_1 = \frac{1}{k_{PFS}(P)}\left(1 + \frac{K_{PFS}}{(S)}\right) \qquad (10)$$

and

$$b_1 = \frac{1}{k_{PFS}(P)} \cdot \frac{K_{PFS}K_{PF}}{(S)} \qquad (11)$$

Equation 9 can be tested experimentally, and at constant pH there should be found the following. (1) At a given value of (S) a straight line relation (Equation 9) exists between $1/V$ and $1/(f)$. The intercept constant is a_1 and the slope is b_1 and hence both can be evaluated at the given value of (S). (2) Furthermore (Equation 10) the values of a_1 obtained at different values of (S) should be a straight line function of $1/(S)$. (3) Likewise (Equation 11) the different values of b_1 should be a straight line function of $1/(S)$. That these expectations are experimentally realized can be seen from Figs. 1 to 4. In Figs. 1 and 4 are shown the experimental data obtained with an oxidase preparation at different concentrations of $d(-)$-alanine and at two values of pH. In each case $1/V$ plotted against $1/(f)$ falls with sufficient accuracy about a straight line. The intercept constants (a_1) and the slopes (b_1) are different for each value of (S), as would be expected. When the intercept constants (a_1) are plotted against the values of $1/(S)$, as in Fig. 2, a straight line relation is found. Also when the slopes (b_1) are plotted as a function of $1/(S)$, as in Fig. 3, a similar linear relation is found. Since these findings are in accord with the anticipations

from Equations 9, 10, and 11, it may be concluded that the theory is in substantial agreement with the experimental data.

Calculation of Dissociation Constants K_{PFS} and K_{PP} from Kinetic Data— From Equation 10, it is clear that the intercept constant in Fig. 2 gives the value of $k_{PFS}(P)$ for the system. Further, the slope, $da_1/d(1/(S))$, is $K_{PFS}/k_{PFS}(P)$. From these two values it is possible to calculate K_{PFS} which is found to be 0.0045. According to Equation 4, this is the concentration of $d(-)$-alanine at which *that portion of the total enzyme which is combined with FAD is also half combined with the amino acid.* According to Equation 11 the intercept constant in Fig. 3 should be zero. This
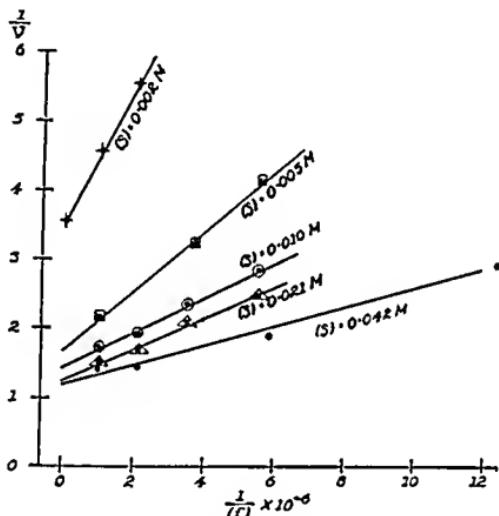
FIG. 1. Velocity of reaction of amino acid oxidase with $d(-)$-alanine. $t = 38°$; pyrophosphate buffer, pH 7.5; 0.3 mg. of oxidase preparation in 2.4 cc. of buffer; flavin adenine dinucleotide of purity 0.20. $1/V$ = reciprocal micromoles of oxygen per minute per mg. of oxidase preparation; $1/(f)$ = reciprocal mole per liter of free FAD; (S) = mole per liter of $d(-)$-alanine.

has been found to be approximately so in our experiments. Further, the slope, *i.e.* $db_1/d(1/(S))$, is $K_{PFS}K_{PP}/k_{PFS}(P)$. Since $k_{PFS}(P)$ and K_{PFS} are known from the data on a_1 *versus* $1/(S)$, it is possible to calculate $K_{PFS}K_{PP}$ and hence K_{PP}. These are found to be 2.7×10^{-9} and 0.6×10^{-6} respectively. From Equation 3 it is apparent that when the concentration of FAD is equal to K_{PP}, *i.e.* $= 0.6 \times 10^{-6}$ mole per liter, *the enzyme other than that combined with the substrate is half combined with the coenzyme.* These constants are analogous to the Michaelis-Menten constant. But instead of indicating the concentration at which the enzyme is half saturated, it is easy to show from Equations 3, 4, and 5 that when the concentrations of flavin and $d(-)$-alanine are 0.6×10^{-6} and 0.0045 mole per liter respec-
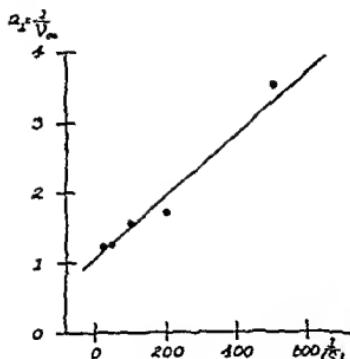
FIG. 2   FIG. 3

FIG. 2. Calculation of equilibrium constants from kinetic data. pH 7.5. $a_1 = 1/V_\infty =$ reciprocal micromoles of oxygen per minute per mg. of (impure) oxidase; $1/(S) =$ reciprocal moles per liter of $d(-)$-alanine.

FIG. 3. Calculation of equilibrium constants from kinetic data. pH 7.5. $b_1 = d(1/V)/d(1/(f)) =$ the slope of the lines of Fig. 1 at a given concentration of $d(-)$-alanine; $1/(S) =$ reciprocal moles per liter of $d(-)$-alanine.
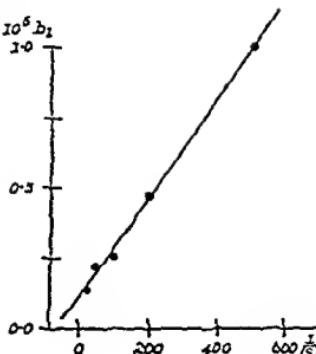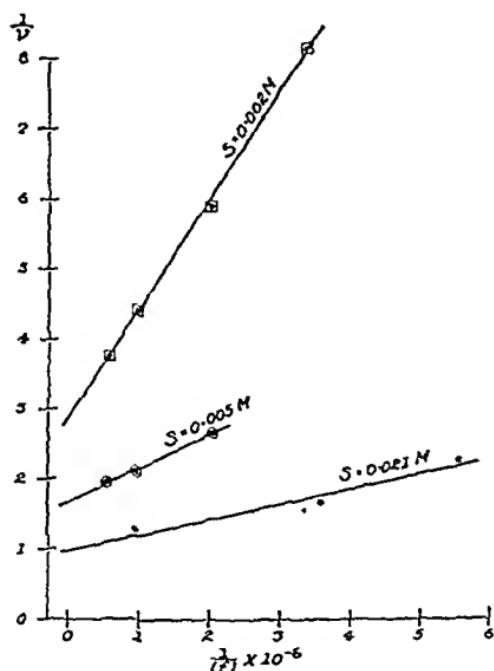
FIG. 4. Velocity of reaction of amino acid oxidase with $d(-)$-alanine. $t = 38°$; pyrophosphate buffer at pH 8.4; 0.3 mg. of oxidase (impure) preparation in 2.4 cc. of buffer; flavin adenine dinucleotide of purity 0.20. $1/V =$ reciprocal micromoles of oxygen per minute per mg. of (impure) oxidase preparation; $1/(f) =$ reciprocal mole per liter of free FAD; $(S) =$ mole per liter of $d(-)$-alanine.

tively, *i.e.* equal to the respective mass action constants, K_{PF} and K_{PFS}, there is calculated the following: $(p) = (PF) = (PFS) = \frac{1}{3}(P)$. That is to say, the enzyme is equally distributed among the three forms; *viz.* uncombined enzyme, enzyme combined with FAD, and enzyme combined with enzyme *and* substrate. Further, at the concentrations being considered, Equation 8 gives $V = \frac{1}{3}k_{PFS}(P)$. But since $k_{PFS}(P)$ is the maximum possible velocity attained when all of the enzyme exists as PFS, *i.e.* (*f*) and (*S*) are very large, we have, when $S = K_{PFS}$ and $(f) = K_{PF}$, $V = \frac{1}{3}V_{\text{maximum}}$. This relation is to be contrasted with the relation obtained from the Michaelis-Menten constant in cases of enzyme systems which require no coenzyme. In this case when the substrate concentration $= K$ (Michaelis-Menten constant), $V = \frac{1}{2}V_{\text{maximum}}$. It is possible then by means of two mass action constants, K_{PFS} and K_{PF}, and one stoichiometrical constant, $k_{PFS}(P)$, to characterize the kinetic behavior of an enzyme which like amino acid oxidase requires a coenzyme in order to react with its substrate.

Calculation of Concentration of Free FAD—Equation 7, 8, or 9 requires that the concentration of free FAD, (*f*), be known. This can be calculated with sufficient accuracy from the known value of the concentration of total FAD, (*F*), in the following way.

From Equations 4 and 6

$$(f) = (F) - [(PF) + (PFS)] = (F) - \left[1 + \frac{K_{PFS}}{(S)}\right](PFS) \qquad (12)$$

The approximate concentration of (*PFS*), the active enzyme-flavin-substrate complex, can be calculated from the experimental findings of Negelein and Brömel (4). At 38°, with a small amount of highly purified amino acid oxidase together with relatively large amounts of FAD and *dl*-alanine in pyrophosphate at pH 8.3, they measured the rate of uptake of oxygen. Under these conditions the enzyme would be completely saturated with both coenzyme and substrate; that is, would be all *PFS*. Hence the velocity would be maximum and would be a measure of the amount of enzyme in the system. Assuming a molecular weight of 70,000, they estimate on the basis of these experiments that 1 gm. mole of enzyme of purity 1 would cause the uptake at 38° of 2000 gm. moles of oxygen per minute. Hence in any similar system the uptake of oxygen in moles per minute divided by 2000 gives the moles of pure enzyme as (*PFS*) in the system. In the experiments given here the oxygen uptake (*V*) has been calculated to micromoles per mg. of (impure) oxidase preparation per minute. Since 0.3 mg. of oxidase preparation in 2.4 cc. was always used, the conversion

of the observed V to moles of (PFS) per liter is given by the conversion equation

$$(PFS) = \frac{0.3V \times 10^{-6}}{0.0024} \times \frac{1}{2000} = 0.06V \times 10^{-6} \text{ mole per liter} \tag{13}$$

From a preliminary plot of the values of $1/V$ as a function of the reciprocal concentration of total FAD (*i.e.*, $1/(F)$) a first approximation of K_{PFS} is made. With this value of K_{PFS}, the values of (PFS) and hence of (f) for each value of V can be calculated by means of Equations 12 and 13. A second approximation (which usually suffices) of K_{PFS}, (PFS), and (f) can then be similarly made. Since in all cases the correction applied to (F) to calculate (f) never exceeded 15 per cent of the total, the approximations used in this calculation result in no serious error in the final calculations of the constants.

Relation of $K_{PFS}K_{PF}$ to K of Warburg and Christian—Warburg and Christian (5) determined the velocity of uptake of oxygen at 38° in a system composed of 2.4 cc. of sodium pyrophosphate at pH 8.3, 20 γ of a highly purified (purity approximately 0.7) amino acid oxidase, and a preparation of 100 per cent pure FAD. From their results they calculated a constant which they defined as follows:

$$K = c \cdot \frac{\text{protein}_{frei}}{\text{protein}_{gebunden}} = c \cdot \frac{v_B - v}{v}$$

where $c =$ the concentration of *total* FAD, $v =$ the observed velocity, and $v_B =$ the maximum velocity when c is very large. Transformation of this equation algebraically gives

$$\frac{1}{v} = \frac{1}{v_B} + \frac{1}{v_B} \cdot \frac{K}{c} \tag{14, a}$$

This equation has the same form as Equation 8 except that the concentration of total FAD (c) is given instead of the concentration of the free flavin (f). By the method of indeterminate coefficients the two equations (Nos. 8 and 14, a) give

$$\frac{1}{v_B} \cdot \frac{K}{c} = \frac{1}{k_{PFS}(P)} \frac{K_{PFS}K_{PF}}{(S)(f)} \tag{14, b}$$

Warburg and Christian, using the concentration of *total* flavin, calculated K to be 0.196 γ per cc. or 0.25×10^{-6} mole per liter. More correctly, however, Warburg and Christian's equation should contain (f), the concentration of *free* FAD, rather than (c), the concentration of *total* FAD; *i.e.*, $c = (f)$. Furthermore, v_B is the maximum velocity when $(S) = 0.021$ and (f) is very large, but $k_{PFS}(P)$ is the maximum velocity when both

(S) and (f) are very large. However, these values do not differ greatly and as a sufficient approximation we take $v_E = k_{PFS}(P)$. Then Equation 14, b becomes

$$K_{PFS}K_{PF} = (S)K \qquad (14, c)$$

We have recalculated Warburg and Christian's data using the values of (f) calculated by the method previously described. We obtain $K =$
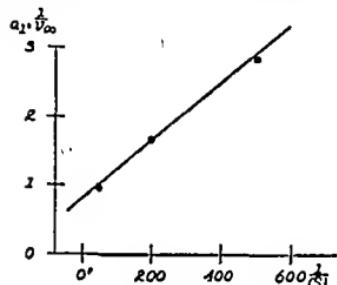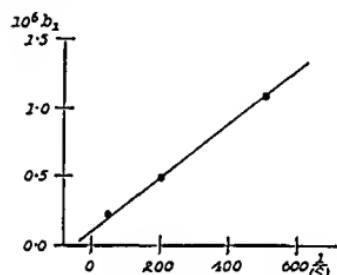
FIG. 5   FIG. 6

FIG. 5. Calculation of equilibrium constants from kinetic data. pH 8.4. $a_1 = 1/V_\infty$ = reciprocal micromoles of oxygen per minute per mg. of (impure) oxidase; $1/(S)$ = reciprocal moles per liter of $d(-)$-alanine.

FIG. 6. Calculation of equilibrium constants from kinetic data. pH 8.4. $b_1 = d(1/V)/d(1/(f))$ = the slope of the lines of Fig. 4 at a given concentration of $d(-)$-alanine; $1/(S)$ = reciprocal moles per liter of $d(-)$-alanine.

TABLE I

Mass Action Constants of the System Amino Acid Oxidase, Flavin Adenine Dinucleotide, $d(-)$-Alanine Calculated from Kinetic Data

Author	pH	K_{PFS}	$K_{PF}K_{PFS}$ $\times 10^9$	$K_{PF} \times 10^6$
		mole per l.	moles per l.	mole per l.
Stadie and Zapp....	7.5	0.0045	2.7	0.60
" " "	8.4	0.0053	2.5	0.47
Warburg and Christian (5)	8.4		3.0	

0.14×10^{-6} mole per liter. It is to be noted that this value is considerably different from the value of $K = 0.25 \times 10^{-6}$ calculated with c, the total FAD concentration. For the value of $(S) = 0.021$ M this gives, by Equation 14, c, $K_{PFS}K_{PF} = 3.0 \times 10^{-9}$ mole per liter. This is to be compared with our own value at pH 8.4 calculated from the data (Figs. 5 and 6) giving the variations of $1/V$ with $1/(f)$ at varying values of $1/(S)$, and the variations of a_1 and b_1 with $1/(S)$. The value of $K_{PFS}K_{PF}$ obtained is 2.5×10^{-9}; i.e., in substantial agreement with the value calculated from Warburg and Christian's data.

It is to be noted that the constant used by Warburg and Christian is strictly not a mass action constant, since it contains a concentration term (S).

The complete data are summarized in Table I.

Case II. Effect of Varying pH When Oxidase Is Completely Saturated with FAD (i.e. (f) Very Large) and Substrate Concentration (S) Is Less Than Saturation Value

Terminology

(P) = total concentration of oxidase, moles per liter

$(PFZ^{\pm})$ = concentration of oxidase-flavin compound as zwitter ion, moles per liter

$(PFZS^{\pm})$ = concentration of corresponding compound of zwitter ion with $d(-)$-alanine, moles per liter

(PFA^{-}) = concentration of ionized oxidase-flavin compound, moles per liter

$(PFAS^{-})$ = concentration of corresponding ionized compound with $d(-)$-alanine, moles per liter

$K_{PFZS}, K_{PFAS}, K_{ac}$ = dissociation constants

$k_{p/zs}, k_{p/as}$ = velocity constants

When the concentration of FAD is many times the value of K_{PF} all of the oxidase is combined with the FAD. But if (S) is less than the saturation value, part of the oxidase-FAD complex will be combined with substrate and part not. In addition, if the effect of pH is to be evaluated, it is necessary to assume that the respective enzyme components will exist (on the alkaline side of the isoelectric point) in part as zwitter ions and in part as protein anions. Then the concentration of total enzyme is given by the equation

$$(P) = (PFZ^{\pm}) + (PFZS^{\pm}) + (PFA^{-}) + (PFAS^{-}) \tag{15}$$

Under these conditions it is possible to assume that the following equilibria exist.

Reaction	Mass action relation	
$PFZS^{\pm} = PFZ^{\pm} + S$	$\dfrac{(PFZ^{\pm})(S)}{(PFZS^{\pm})} = K_{PFZS}$	(16)
$PFAS^{-} = PFA^{-} + S$	$\dfrac{(PFA^{-})(S)}{(PFAS^{-})} = K_{PFAS}$	(17)

In order to evaluate the effect of changing pH upon the kinetics of the oxidation of the amino acid it is necessary to assume that the various forms of the enzyme are in equilibrium with hydrogen ions. Only one mass action relation need be set up, however, and for this purpose we choose the

active form of the oxidase-flavin-substrate compound; *viz.*, $PFZS^{\pm} = PFAS^- + H^+$, for which the mass action equation is

$$\frac{(PFAS^-)(\text{H}^+)}{(PFZS^{\pm})} = K_{ac} \tag{18}$$

The combination of Equations 15, 16, 17, and 18 give

$$(P) = \left[1 + \frac{K_{ac}}{(\text{H}^+)} + \frac{K_{PFZS}}{(S)} + \frac{K_{PFAS}K_{ac}}{(S)(\text{H}^+)} \right](PFZS^{\pm}) \tag{19}$$

But it is known that the over-all velocity of the oxidative reaction varies with pH and it may be assumed that this is owing to the fact that the "active" forms $PFZS^{\pm}$ and $PFAS^-$, which change with pH, have different velocity constants. We may therefore equate the total velocity as follows:

$$V = k_{pfzs}(PFZS^{\pm}) + k_{pfas}(PFAS^-) \tag{20}$$

Eliminating $(PFAS^-)$ from this equation by means of Equation 18, we obtain

$$V = \left[k_{pfzs} + k_{pfas} \frac{K_{ac}}{(\text{H}^+)} \right](PFZS^{\pm}) \tag{21}$$

Finally, the combination of Equations 19 and 21 may be put into the form most convenient for calculation with the elimination of $(PFZS^{\pm})$; *viz.*,

$$\frac{1}{V} = \frac{1}{k_{pfzs}(P)} \left[\frac{K_{ac} + (\text{H}^+)}{\dfrac{k_{pfas}}{k_{pfzs}} K_{ac} + (\text{H}^+)} + K_{PFZS} \frac{\dfrac{K_{PFAS}}{K_{PFZS}} K_{ac} + (\text{H}^+)}{\dfrac{k_{pfas}}{k_{pfzs}} K_{ac} + (\text{H}^+)} \cdot \frac{1}{(S)} \right] \tag{22}$$

This equation, for ease of reference and calculation, is put in the form

$$\frac{1}{V} = a_2 + \frac{b_2}{(S)} \tag{23}$$

where

$$a_2 = \frac{1}{k_{pfzs}(P)} \left(\frac{K_{ac} + (\text{H}^+)}{\dfrac{k_{pfas}}{k_{pfzs}} K_{ac} + (\text{H}^+)} \right) \tag{24}$$

$$b_2 = \frac{1}{k_{pfzs}(P)} \cdot K_{PFZS} \cdot \frac{\dfrac{K_{PFAS}}{K_{PFZS}} K_{ac} + (\text{H}^+)}{\dfrac{k_{pfas}}{k_{pfzs}} K_{ac} + (\text{H}^+)} \tag{25}$$

It will be shown that the experimental data are in conformity with the expectations from Equations 23, 24, and 25. The method of calculating the constants involved in these equations will be illustrated.

EXPERIMENTAL

Preparations of amino acid oxidase (0.3 mg. in 2.4 cc. of buffer) were mixed with sufficiently large amounts of FAD to assure saturation. At constant pH but with five to six different concentrations of $d(-)$-alanine the velocity of oxidation of the amino acid as measured by the rate of
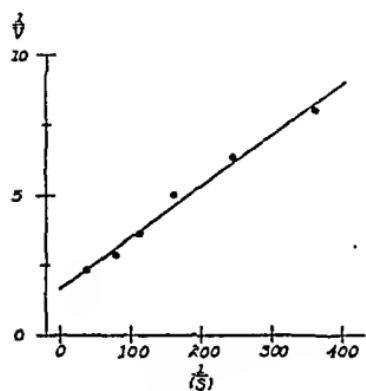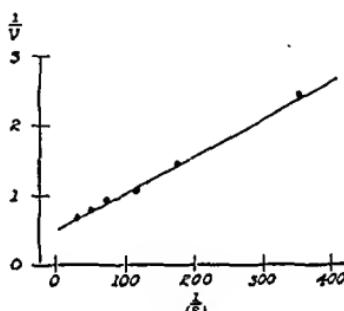
FIG. 7. Velocity of oxidation of $d(-)$-alanine by amino acid oxidase fully saturated with flavin adenine dinucleotide at pH 6.5. $1/V$ = reciprocal micromoles of oxygen per minute per mg. of (impure) oxidase preparation; $1/(S)$ = reciprocal moles per liter of $d(-)$-alanine.

FIG. 8. Velocity of oxidation of $d(-)$-alanine by amino acid oxidase fully saturated with flavin adenine dinucleotide. pH 9.0. $1/V$ = reciprocal micromoles of oxygen per minute per mg. of (impure) oxidase preparation; $1/(S)$ = reciprocal moles per liter of $d(-)$-alanine.

oxygen uptake was determined at 38° as described before. The results were calculated as micromoles of oxygen per mg. of oxidase preparation per minute. These observations were repeated over selected values of pH ranging from 6.5 to 9.0.

Two typical individual experiments are shown in Figs. 7 and 8 at pH 6.5 and 9.0 respectively. As anticipated from Equation 23, there was found a straight line relation between $1/V$ and $1/(S)$ in each case.

From the straight line it is possible to calculate a_2, the value of the intercept constant (when $1/(S) = 0$), and also b_2, the slope of the line. Altogether eight such experiments were done, each with five to six different values of $d(-)$-alanine. In all cases similar linear relations were found between $1/V$ and $1/(S)$.

The values of $1/a_2$ and b_2 calculated from these lines are plotted in Figs. 9 and 10 respectively over the pH range 6.5 to 9.0. It will be shown, in each case, that the form of the curve is that anticipated from the theory.
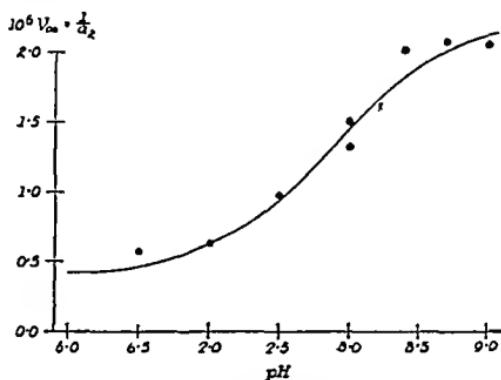
FIG. 9. Calculation of equilibrium constants of amino acid oxidase-flavin adenine dinucleotide and $d(-)$-alanine at varying pH from kinetic data. The points are experimentally determined from the intercept constants of the relation $1/V = a_2 + (b_2/(S))$ at varying pH. The curve is theoretically calculated (see the text). $V_\infty = 1/a_2 =$ reciprocal micromoles of oxygen per minute per mg. of oxidase preparation.
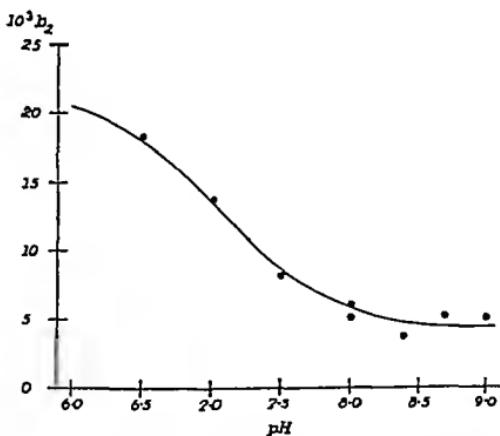
FIG. 10. Calculation of equilibrium constants of amino acid oxidase-flavin adenine dinucleotide and $d(-)$-alanine at varying pH from kinetic data. The points are experimentally determined from the slopes of the equation $1/V = a_2 + (b_2/(S))$ at varying pH. The curve is theoretically calculated (see the text).

Calculations of Constants of Equation 22 from Values of a_2 and b_2 at Varying pH—This can be accomplished by the use of familiar mathematical devices using Equations 24 and 25, as follows:

1. From Equation 24 when (H^+) is very large with respect to K_{ac} and $(k_{pfas}/k_{pfzs})K_{ac}$, we obtain

$$\frac{1}{a_2} = k_{pfzs}(P) \tag{26}$$

We would expect then that $1/a_2$ would approach a limiting minimum value as the pH^+ is decreased. This is found to be the case, as Fig. 9 shows. Hence $k_{pfzs}(P)$ can be evaluated from the experimental data.

2. When (H^+) is very small with respect to the same constants, we have

$$\frac{1}{a_2} = k_{pfas}(P) \tag{27}$$

Likewise we would expect a limiting maximum value of $1/a_2$ as the pH^+ is increased. This is also (Fig. 9) found to be the case. Similarly, then $k_{pfas}(P)$ can be evaluated.

3. Differentiating Equation 24 with respect to pH, we obtain

$$\frac{d\left(\dfrac{1}{a_2}\right)}{d\,pH} = 2.3[k_{pfas}(P) - k_{pfzs}(P)]K_{ac}\frac{(H^+)}{[(H^+) + K_{ac}]^2} \tag{28}$$

It is easy to show that this function is a maximum when $(H^+) = K_{ac}$. At that pH^+ where $(H^+) = K_{ac}$ this equation becomes

$$\frac{d\left(\dfrac{1}{a_2}\right)}{d\,pH} = \frac{2.3}{4}[k_{pfas}(P) - k_{pfzs}(P)] \tag{28, a}$$

4. In a similar fashion, when (H^+) is large with respect to $(K_{PFAS}/K_{PFZS})K_{ac}$ and $(k_{pfas}/k_{pfzs})K_{ac}$, Equation 25 becomes

$$b_2 = \frac{K_{PFZS}}{k_{pfzs}(P)} \tag{29}$$

We would expect b_2 to approach a limiting maximum value as pH is decreased. This expectation was not fully realized experimentally (Fig. 10), because it was not possible to work at a sufficiently acid pH to reach the maximum. A sufficient approximation, however, can be established.

5. When (H^+) is small with respect to the same constants

$$b_2 = \frac{K_{PFAS}}{k_{pfas}(P)} \tag{30}$$

Again a limiting minimum value of b_2 at high pH^+ should be found. Experimentally this was realized, as Fig. 10 shows.

6. Likewise by differentiating b_2 with respect to pH there is found

$$\frac{db_2}{d\,\mathrm{pH}} = 2.3\left[\left(\frac{K_{PFAS}}{k_{pfas}(P)} - \frac{K_{PFZS}}{k_{pfzs}(P)}\right)\cdot\frac{(\mathrm{H^+})\dfrac{k_{pfas}}{k_{pfzs}}K_{ac}}{\left((\mathrm{H^+}) + \dfrac{k_{pfas}}{k_{pfzs}}K_{ac}\right)^2}\right] \qquad (31)$$

It is easy to show that this function is a maximum when $(\mathrm{H^+}) = (k_{pfas}/k_{pfzs})K_{ac}$. The function then becomes

$$\frac{db_2}{d\,\mathrm{pH}} = \frac{2.3}{4}\left[\frac{K_{PFAS}}{k_{pfas}(P)} - \frac{K_{PFZS}}{k_{pfzs}(P)}\right] \qquad (31,\,a)$$

At the middle range of pH of Figs. 9 and 10, where the slopes are essentially constant, the values can be applied to the respective Equations 28, a and 31, a.

TABLE II

Mass Action Constants for Amino Acid Oxidase Calculated from Kinetic Data at Varying pH

	From $\frac{1}{a_2}$ versus pH	From b_2 versus pH
$k_{pfzs}(P) = 0.39^*$		
$k_{pfas}(P) = 2.3^*$		
k_{pfas}/k_{pfzs}	5.9	
K_{PFZS}		0.0087
K_{PFAS}		0.0092
pK_{ac}	7.90	7.82

* No physicochemical significance except in the ratio.

We therefore have six independent equations, viz. Equation 26, 27, 28, 29, 30, and 31, containing five unknowns, viz. $k_{pfzs}(P)$, $k_{pfas}(P)$, K_{PFZS}, K_{PFAS}, and K_{ac}. There are also six experimentally determined quantities; viz., four intercept constants and two slopes. It is therefore theoretically and practically possible to evaluate all of the unknown constants and indeed one may be evaluated twice.

In practice it is easy, in view of the above relations, to calculate by two or three approximations constants which best fit the experimental data. The values selected are given in Table II. Using these values, we calculated the full lines shown in Figs. 9 and 10. We regard the fit of the theoretical line with the experimental points to be sufficiently good to warrant the conclusion that the hypotheses upon which the theory is based form a reasonable explanation of the kinetics of amino acid oxidase fully saturated

But, as before, the velocity of the reaction is proportional to the active form of the enzyme; *i.e.*, $V = k(PFS)$. But the maximum velocity (*i.e.* when (S) is very large) *at the given* pH is proportional to the total enzyme; *i.e.*, $V_{(S=\infty)} = k(P)$. Whence

$$\frac{1}{V} = \frac{1}{V_{(S=\infty)}}\left(1 + \frac{K_m}{(S)}\right)$$

Comparison of this form of the Michaelis-Menten equation with Equation 32 shows at once that

$$V_{(S=\infty)} = k_{pfzz}(P)\frac{\dfrac{k_{pfaz}}{k_{pfzz}}K_{ac} + (\text{H}^+)}{K_{ac} + (\text{H}^+)} \tag{33}$$

Also $K_{PS} = K_m$.

This relation signifies that at any given pH the customary Michaelis-Menten equation (No. 33) is a special case of the more general equation (No. 32). The maximum velocity observed at any given pH is a function of the acid dissociation constant of the enzyme, the velocity constants of the zwitter ion and anion form of the enzyme, and the hydrogen ion concentration. The Michaelis-Menten constant is, as would be anticipated, the dissociation constant of the enzyme-substrate complex.

The theoretical treatment of the effect of changing pH in Case I, *i.e.* when both coenzyme and substrate are less than the saturation values, can also be accomplished along the same lines as that used in Case II. However, the difficulties in performing the experiments over a sufficiently wide range of pH resulted only in partial success in testing the theory.

In Equation 24, $1/a_2 = V_{(S=\infty)}$ is the velocity of the reaction when (S) is very large and hence is the *maximum velocity at that pH*. Three possible types of enzymes are conceivable, each giving variants in the behavior of this velocity with respect to pH. These types are apparent from a consideration of Equation 28 which can also be derived directly from Equation 33; *viz.* (1) The anion form of the enzyme-substrate complex is catalytically more active than the zwitter ion form; *i.e.*, $k_{pfaz} > k_{pfzz}$. Then $V_{(S=\infty)}$ is a minimum at low pH, a maximum at high pH, and follows a curve of intermediate values similar to that in Fig. 9. (2) The zwitter ion is the more active form; *i.e.*, $k_{pfaz} < k_{pfzz}$. Then, conversely, $V_{(S=\infty)}$ is a minimum at high pH and a maximum at low pH. (3) There is no difference in the activity of the two forms; *i.e.*, $k_{pfaz} = k_{pfzz}$. In that case, pH has no effect upon $V_{(S=\infty)}$. Whether all these forms exist in reality remains to be seen.

Finally, it must be remembered that pH may affect by ionization the forms of the substrate and hence its equilibrium with the forms of the en-

zyme. In that case, the theoretical treatment becomes more complex. In the case of the oxidation of $d(-)$-alanine by d-amino acid oxidase, this plays no rôle except at pH values greater than 9.0. Hence this factor has been omitted in the discussions here.

SUMMARY

1. The velocity of the oxidation of $d(-)$-alanine in the presence of d-amino acid oxidase and flavin adenine dinucleotide was determined under varying conditions.

2. A series of determinations was carried out at constant pH and constant substrate concentration, but varying flavin adenine dinucleotide concentrations, but all less than saturation concentration. A theoretical equation was formulated on the basis of simple assumptions expressing the velocity in terms of equilibrium constants. The experimental data were found to be in substantial agreement with the theory, and from the kinetic data the equilibrium constants were calculated.

3. A second series of determinations was carried out in which the flavin adenine dinucleotide was in sufficient excess to saturate the enzyme. Data were obtained over a wide range of substrate concentration and pH. In this case, also, on the basis of simple assumptions the velocity of oxidation was expressed theoretically as a function of equilibrium constants including the acid dissociation constant of the enzyme. The experimental results were in agreement with the predictions of the theory. Hence the equilibria constants in this case could be calculated from the kinetic data.

4. The theory and experimental data permit an extension of the Michaelis-Menten concept of enzyme action to include the equilibria involving enzyme, coenzyme, substrate, and hydrogen ions.

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THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

III. ADENOSINETRIPHOSPHATASE*

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Adenosine triphosphate (ATP) is important in cellular economy because it possesses the ability to store within its structure the energy which is made available by the processes of anaerobic (1) as well as aerobic metabolism (2–5). So far as is now known, no other compound is able to react directly with these glycolytic and respiratory processes and to capture the energy which these processes release.

There is also considerable evidence that one of the prime functions of ATP is the activation of the substrates of glycolysis. This is done in a number of ways. By means of the enzyme hexokinase (6, 7), glucose is converted to glucose-6-phosphate by means of the phosphate energy of ATP. The conversion of glucose-6-phosphate to fructose diphosphate likewise requires the ATP system for its completion (1). Furthermore, two steps in glycolysis require inorganic phosphate, which becomes esterified, and two require adenylic acid as a phosphate acceptor (1). Both inorganic phosphate and adenylic acid are products of the breakdown of ATP.

Although it might be thought that the ATP system exists for the sole purpose of activating the processes of glycolysis and respiration, this view was weakened considerably by the work of Colowick, Kalckar, and Cori (4), in which it was shown that the complete combustion of 1 mole of glucose led to the esterification of 10 moles of inorganic phosphate *in excess of that required to activate the glucose.* These authors expressed the view that in the normal cell this esterified phosphate must be diverted to a variety of other cellular functions, instead of accumulating as hexose diphosphate. One might assume *a priori* that since glycolysis and respiration had been shown to be coupled with phosphate esterification the energy of the ATP molecule must be usable for all forms of metabolic work. In the case of muscle such an assumption seems no longer necessary, since strong evidence has come from several laboratories indicating that myosin, the contractile element of muscle, is either identical with adenosinetriphosphatase or is intimately bound to it (8–10). Adenosinetriphosphatase catalyzes the following reaction.
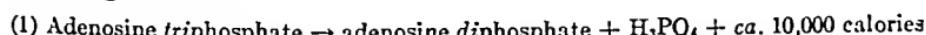
(1) Adenosine *tri*phosphate $\rightarrow$ adenosine *di*phosphate $+ H_3PO_4 + ca.$ 10,000 calories

This reaction is so exergonic (Coryell terminology (11)) that it is for all practical purposes irreversible, and it is probable that adenosinetriphosphatase has nothing to do with the reversal of the reaction. In the intact muscle cell the energy is used to do muscular work; that is, the shortening of the myosin fiber is somehow associated with the release of inorganic phosphate from ATP. In a solution of myosin, no muscular work is performed; yet ATP can be split to yield ADP and inorganic phosphate (10). We assumed that if ATP is the source of energy for the metabolic work which *other* tissues perform, these tissues should also contain an adenosinetriphosphatase whose ATP-splitting function could be measured in the absence of the specific function of the tissue involved.

The present paper demonstrates that other tissues in addition to muscle contain adenosinetriphosphatase, and presents a method for the quantitative estimation of this enzyme, based on the principle of diluting tissue homogenates and measuring their activity in the presence of an excess of substrate and cofactors, as has been done previously in this laboratory (12–15). Since the synthesis of ATP is largely derived from the action of the respiratory enzymes, we are including this paper in the series on respiratory enzyme assays.

<div align="center">EXPERIMENTAL</div>

<div align="center">*Method*</div>

After study of the optimum concentration of each of the components of this enzyme system the following assay method was developed, in which the quantity of adenosinetriphosphatase determines the velocity of the liberation of inorganic phosphorus from ATP. To small test-tubes (10 × 50 mm.) are added 0.15 ml. of 0.05 M diethyl barbiturate buffer (pH 7.4), 0.05 ml. of 0.04 M $CaCl_2$, and 0.15 ml. of 0.013 M ATP neutralized to pH 7.4. Enough distilled water is added so that the final volume is 0.65 ml. after the tissue is added. The final volume is small in order to decrease the quantity of substrate necessary for each determination. Pipettes of 0.1 and 0.2 ml. capacity, graduated in hundredths, and having the tips drawn out to a thin walled capillary, are used to measure the reagents. The tissue homogenates are freshly prepared by homogenizing the tissues in cold distilled water. A 10 per cent homogenate is usually prepared and diluted to 1 per cent with cold distilled water for use, thus facilitating the addition of 1 or 2 mg. of tissue to the reaction mixture, which is placed in the thermostat a few minutes before the tissue addition. After addition of the tissue, the samples are incubated at 37° for 15 minutes. At the end of the incubation period the reaction is stopped by the addition of 0.1 ml. of 50 per cent trichloroacetic acid. The mixture is centrifuged at 3000 R.P.M. for 10 minutes and 0.3 ml. of the supernatant is carefully removed for analysis.

The inorganic phosphorus liberated from ATP is measured in a Cenco photoelectric spectrophotometer by the method of Fiske and Subbarow (16), a final volume of 3 ml. being used. An Evelyn colorimeter may be used if 0.6 ml. of supernatant in a final volume of 6.0 ml. is employed. The inorganic phosphorus in the tissue homogenates and in the ATP is always determined and subtracted from the inorganic phosphorus value obtained for the incubation mixture. Instead of determining the activity on duplicate tissue samples of the same weight, we always use different concentrations of tissue. Therefore in every determination there is assurance that the phosphate liberated is proportional to the adenosinetriphosphatase activity. The quantity of adenosinetriphosphatase which will liberate 1 γ of inorganic phosphorus from ATP in 15 minutes at 37° is called 1 adenosinetriphosphatase unit. The values are expressed as the number of units of adenosinetriphosphatase per mg. of fresh tissue. Parallel determinations are also carried out without calcium added. When no calcium is added, the amount of phosphorus liberated from ATP is partly dependent upon the amount of calcium in the tissue homogenate. Such a determination, therefore, gives an indication of the amount of tissue calcium which is in combination with adenosinetriphosphatase.

Comments on Method

Two requirements which should be fulfilled in any enzyme assay method in which rate measurements are used as the index of enzyme concentration are the following, (a) that the rate of the reaction is linear during the time of incubation and (b) that the rate is directly proportional to the amount of enzyme present in the test system. That the method outlined above meets those requirements is demonstrated in Figs. 1 and 2. 1 mg. each (wet weight) of brain, kidney, skeletal muscle, and liver tissue was used in the experiments reported in Fig. 1, which shows that the hydrolysis of ATP was proportional to the time of incubation over a period of 30 minutes in the case of skeletal muscle, kidney, and liver. With brain tissue (Curve D) the hydrolysis was proportional to the time of incubation for only the first 20 minutes. This decrease in activity after 20 minutes incubation in the case of brain tissue cannot be ascribed to a deficiency of substrate or coenzyme, since a linear relationship between time and rate was obtained with other tissues in which the enzyme concentration was greater. It appears that some substance liberated during incubation of brain tissue inhibits the activity of adenosinetriphosphatase. This substance may be the same as the inhibitor of glycolysis in brain extracts, which has been studied by Geiger (17, 18) and Ochoa (7). The amount of ATP split by the various tissue homogenates varied directly with the incubation time for at least 15 minutes, which was chosen as the standard time for the assay.

That the test system meets the second requirement is demonstrated in

Fig. 2. The effect of varying the quantity of tissue was tested on four tissues by means of the method described except that the ATP solution was 0.01 M instead of 0.013 M. Fig. 2 shows that the rate was proportional to the tissue concentration for 3 mg. or less of tissue. 0.5 and 1 mg. samples of tissue are used in the assay of tissues in which the activity per mg. is more than 10 adenosinetriphosphatase units. In tissues in which the activity was less than 10 units 1 and 2 mg. of tissue are used for the determination. The decrease in activity with 6 mg. of tissue was shown to be due to a lack of sufficient substrate, since when the substrate concentration was doubled (Curve C') the activity of liver tissue was proportional to the tissue con-
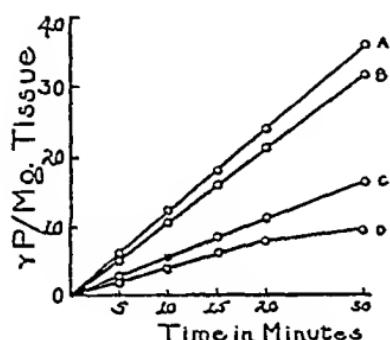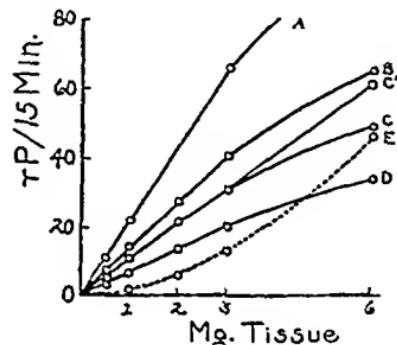
FIG. 1. The effect of different incubation times on the hydrolysis of ATP. Rat tissues homogenized in cold distilled water were used as the source of the enzyme. Curve A skeletal muscle, Curve B kidney, Curve C liver, Curve D brain.

FIG. 2. The hydrolysis of ATP with different quantities of tissue. Curve A skeletal muscle, Curve B kidney, Curve C liver, Curve D brain, Curve C' liver as in Curve C except that 0.15 ml. of 0.02 M ATP was used instead of 0.01 M ATP, Curve E liver without calcium added to the test system, showing that calcium molarity limits the velocity of the reaction.

centration even when 6 mg. of tissue were used. That skeletal muscle does not show this substrate deficiency as rapidly as liver, brain, or kidney is probably due to the very high quantity of myokinase which it contains (19, 20). This enzyme has been shown to "dismute" 2 moles of ADP to 1 mole of ATP and 1 mole of adenylic acid, which would have the net effect of increasing the ATP concentration.

As shown in Fig. 2, when calcium was not added to the adenosinetriphosphatase system, a dilution effect (12, 13) results. Under these conditions the quantity of tissue calcium which is in combination with adenosinetriphosphatase limits the activity of the system. In the determination without calcium (Curve E), activity is not directly proportional to the tissue concentration because the calcium molarity is the main factor which

determines the rate of reaction and when submaximal quantities of calcium are present the rate is the resultant of two variables and a dilution effect results.

Buffers and pH Optimum—In studying the adenosinetriphosphatase of skeletal muscle Bailey (10) used a 0.1 M potassium chloride-glycine buffer at pH 9.1. He found that a bicarbonate buffer was unsatisfactory due to reaction with the calcium to form insoluble calcium carbonate, thus reducing the effective molarity of the activator. A 0.05 M barbiturate buffer prepared by neutralizing 0.1 M sodium diethyl barbiturate to pH 7.4 with 0.1 M HCl was found to be satisfactory in our work. It seemed desirable to use a pH of 7.4, because that is more nearly physiological and the activity
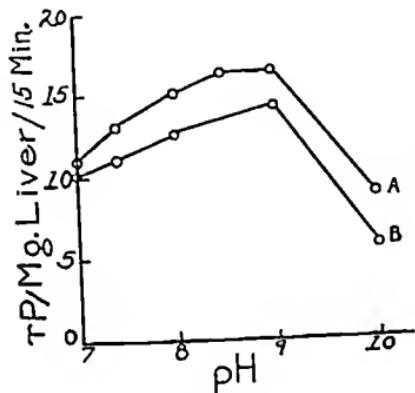
FIG. 3
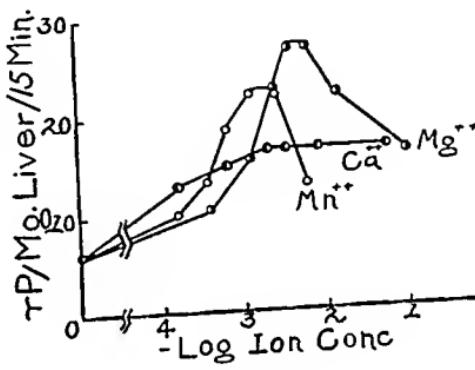
FIG. 4

FIG. 3. The optimum pH for adenosinetriphosphatase activity. Liver tissue was used. Curve A 0.05 M diethyl barbiturate (veronal) buffer (final molarity 0.012), Curve B 0.1 M KCl-glycine buffer (final molarity 0.023).

FIG. 4. Effect of calcium, magnesium, and manganese on the hydrolysis of ATP. A liver homogenate was used as the source of the enzyme. Ions were added as the chlorides. Concentration in moles per liter.

of other phosphatases is at a minimum according to the studies of Woodard (21) on acid and alkaline phosphatases and of Gomori (22) on hexosediphosphatase. If the reaction were carried out at a different pH, the ester phosphatases might attack adenylic acid. Fig. 3 shows the activity of liver adenosinetriphosphatase with barbiturate buffers and potassium chloride-glycine buffers of different hydrogen ion concentrations. A pH of 9.0 was found to be optimum with the activity decreasing rapidly above pH 9.0 and slowly at a pH below 9.0. A potassium chloride-glycine buffer gave the same type of activity curve but is not as good a buffer at pH 7.4. No significance is attached to the difference in activity with the two buffers, since a different rat liver was used for each curve and the results in both cases are within the range usually found for liver.

Optimum Substrate Concentration—ATP was prepared by the method of Needham (9) with the magnesium anesthesia treatment described by DuBois *et al.* (23). The barium salt was carried through the mercury precipitation procedure a second time to reduce to a minimum the amount of inorganic phosphorus in the final preparation. The barium salt was converted to the sodium salt with sodium sulfate before use. In studying the concentration of substrate necessary we found that a final concentration of 0.005 M ATP was necessary for maximum activity of 1 mg. of liver over a period of 60 minutes. However, with a 15 minute incubation period a final molarity of 0.003 M ATP is satisfactory, as is proved in Figs. 1 and 2.

Effect of Calcium, Magnesium, and Manganese—Bailey (10) showed that calcium is the specific activator for the adenosinetriphosphatase of skeletal muscle, that manganese activated to a lesser extent, and that magnesium produced only a slight activation. Lyubimova and Pevsner (24) reported that magnesium inhibits adenosinetriphosphatase of skeletal muscle. It was of interest, therefore, to determine the effect of different concentrations of these three ions on the adenosinetriphosphatase activity of liver tissue.

1 mg. of liver was used as in the test system described above. The quantities of the other constituents of the test system remained constant except the concentration of the activating ion. The effects of these three ions are shown in Fig. 4. A final concentration of 7.7×10^{-4} M calcium was the lowest concentration which would give maximum activation. With magnesium the maximum activation was obtained with 1.5×10^{-3} M but with concentrations above 2.4×10^{-3} M the activity was greatly inhibited. Calcium in similar high concentrations produced no decrease in activity. The effect of the manganese was similar to that of magnesium, producing activation in concentrations up to 3.9×10^{-4} M. The data show that calcium and magnesium have different effects on the activity of adenosinetriphosphatase. In the case of calcium the data suggest that calcium activates the primary reaction (ATP to ADP + H_3PO_4) and that subsequent reactions are quantitatively too small to reveal an inhibiting action of calcium on ester phosphatase. In the case of magnesium, it seems possible that the activation of the myokinase (20) and adenylic acid phosphatase overshadows the inhibition of the adenosinetriphosphatase at those concentrations at which maximum phosphate is split off. At still higher concentrations of magnesium, the inhibition of adenosinetriphosphatase (24) might be sufficient to decrease the amount of adenylic acid available for dephosphorylation, and in this case less inorganic phosphate would be found (see Fig. 4).

Specificity of Adenosinetriphosphatase—Attempts (10) to demonstrate a specific adenosinetriphosphatase in liver tissue have been unsuccessful. Jacobsen (25) studied the dephosphorylation of ATP and his work provides

some evidence for the existence of a specific phosphatase for this hydrolysis. Barrenscheen and Lang (26) also stated that there is a specific enzyme for the dephosphorylation in liver tissue. Sahtoh (27) reported that the complete dephosphorylation of ATP occurs by the combined action of a pyrophosphatase and a phosphomonoesterase, the latter being somewhat specific. Bailey (10) compared the adenosinetriphosphatase activity of liver tissue with that of muscle tissue with calcium as the activator. Although a calcium-activated dephosphorylation of ATP occurred with the liver preparation, it was not as definite as was obtained with muscle adenosinetriphosphatase. Bailey, therefore, concluded that liver did not contain the same adenosinetriphosphatase as muscle tissue. The difference which he obtained was probably due to the different treatment of liver and muscle tissue in the preparation of the enzyme as well as to a difference in concentration of the enzyme in the two tissues.

TABLE I

Specificity of Adenosinetriphosphatase

The final substrate molarity was 0.003 in each case.

Substrate	Adenosinetriphosphatase	
	Calcium added	No calcium added
	units	*units*
ATP..	12.6	2.5
Adenylic acid..........................	2.1	1.8
Hexose diphosphate....................	3.5	3.5
β-Glycerophosphate........................	0.2	0.2

J. Needham *et al.* (28) have studied the rate of hydrolysis of several phosphorus esters by pure adenosinetriphosphatase from muscle. They found that the enzyme catalyzed the liberation of inorganic phosphorus from ATP much faster than from any other compound tested. D. M. Needham (9) found that the enzyme could not attack hexose diphosphate or α-glycerophosphate. It was, therefore, of interest to substitute some organic phosphorus compounds in the test system which we used for determining adenosinetriphosphatase activity. A liver homogenate was used as the source of the enzyme. ATP, hexose diphosphate, adenylic acid, and β-glycerophosphate were tested. Table I shows the comparative rates of hydrolysis of the four compounds under identical conditions. The results clearly indicate that ATP is the only one of these compounds whose dephosphorylation is significantly activated by calcium. The recent report (22) that there is a specific hexosediphosphatase in animal tissues lends support to the probability that phosphatases have specific substrates.

Application of Method—The method which has been described for de-

termination of the quantity of adenosinetriphosphatase in animal tissues has been applied to a number of normal tissues.   Table II gives the adenosinetriphosphatase activity of normal rat tissues.   The values given are the average from five normal rats which all weighed between 250 and 300 gm. and received a normal diet.   The results from individual rats did not differ from the mean by more than 15 per cent.   The high adenosinetriphosphatase content of lung tissue was of particular interest when compared with the concentration of some of the aerobic enzymes in lung tissue. Cytochrome c (29), succinic dehydrogenase, and cytochrome oxidase (15) have been shown to be lower in lung tissue than in other normal tissues.

Table II

Adenosinetriphosphatase Activity of Normal Rat Tissues

The results are expressed in ATP units; *i.e.*, micrograms of inorganic phosphate split off per mg. of fresh tissue per 15 minutes.

Tissue	Calcium added	No calcium added
Cardiac muscle	27.3	4.8
Skeletal "	23.3	12.5
Lung	21.8	3.0
Kidney	20.3	3.5
Submaxillary gland	16.4	3.6
Spleen	13.0	1.4
Liver	12.9	3.9
Pancreas	11.5	1.5
Smooth muscle	8.2	1.9
Brain	7.0	2.4

DISCUSSION

The presence of a calcium-activated adenosinetriphosphatase in various normal tissues raises the question as to the form and functions of the enzyme in these tissues.   Recent investigations (8–10) have shown that in the case of muscle the enzyme activity could not be separated from that of myosin, which suggested that this protein and adenosinetriphosphatase are identical. The experiments reported above show that the adenosinetriphosphatases of tissues other than skeletal muscle exhibit the same *catalytic* properties as that from skeletal muscle; namely, the hydrolysis of ATP in the presence of calcium ions.   However, it is obvious that if this reaction is coupled to the function of the tissue, as we have every reason to assume, the functional component of the enzyme must vary from tissue to tissue, or the adenosinetriphosphatase must be intimately bound to various types of functional proteins in the various specialized tissues.

Although in the test system as used in this study the breakdown of ATP

yields inorganic phosphate directly, it is possible that *in vivo* there is an intermediate step involved, or that the phosphate is merely transferred to some other R protein with the phosphate bond energy falling to the ester level as Lipmann has indicated ((30) pp. 126–127). However, it seems certain that the phosphate eventually reaches the inorganic level ((30) Fig. 1). Whether the ATP energy is used directly or indirectly, it seems reasonable to use the myosin analogy for the present and assume that the lytic action of the adenosinetriphosphatase can be measured in the absence of the specific functions of the various tissues. The experiments reported in this paper do not test the validity of this assumption and therefore leave the question open.[1] Other experiments which do have a bearing on the problem will be reported elsewhere.

If the activity of the adenosinetriphosphatase system is closely associated with the performance of the specific functions of tissues and organs, one might expect that only part of the enzyme is activated at those times when a particular tissue is not working at its maximum capacity. The very fact that both ATP and adenosinetriphosphatase are present in tissues raises the question of how this system is controlled. The adenosinetriphosphatase determinations in tissues with and without added calcium suggest that the quantity of calcium combined with adenosinetriphosphatase at any one time determines the activity of the system. The primary effect of a stimulus may be to make calcium available for the activation of adenosinetriphosphatase (10). Indeed, Heilbrunn has advanced a theory, without reference to any particular enzyme system, that the stimulation of cells in general results in the release of calcium which then produces a tissue response. Evidence that acetylcholine increases adenosinetriphosphatase activity has been reported by DuBois and Potter (31), and the evidence suggested that the effect might be mediated by calcium. The linking of physiological stimuli with the regulation of the rate of glycolysis and the performance of work may well be controlled by the activation of the adenosinetriphosphatase system, since the rate of glycolysis and respiration is influenced by the availability of inorganic phosphate and adenylic acid (1, 32). Studies on the activation and inhibition of the adenosinetriphosphatase system by compounds of known physiological importance should therefore be of con-

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[1] Lipmann's suggestion ((30) p. 149) that "Pyrophosphatase operates rather like an outlet for the adenylic acid system to adjust the flow of ∼ph in case of overproduction, much in the manner of a valve" is probably no longer tenable in view of the subsequent work on myosin, and should therefore not be construed to indicate that the enzyme measured in our work is unrelated to function. As a matter of fact, Lipmann further stated, "In cyclic processes of utilization, we think that to know how inorganic phosphate is recovered would frequently imply knowing the mechanism because complete utilization of the potential energy in ∼ph converts it necessarily into inorganic phosphate."

siderable interest in connection with the specific biochemical action of these compounds.

SUMMARY

1. A method for the determination of adenosinetriphosphatase in animal tissues was developed in which dilute tissue homogenates are used.

2. It was shown that in the test system which was developed the rate of the reaction is linear during the time of incubation and that the rate is directly proportional to the amount of enzyme present in the test system.

3. The adenosinetriphosphatase activity of ten tissues from normal rats was determined, and it was shown that calcium activation occurred in each case.

4. The effect of calcium, magnesium, and manganese on this system was determined.

5. When hexose diphosphate, β-glycerophosphate, or adenylic acid was substituted in the test system for ATP, it was found that ATP was the only compound whose hydrolysis was activated by calcium ions, and that the hydrolysis of the other compounds was very slight under the conditions employed.

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AMIDE METABOLISM IN ETIOLATED SEEDLINGS

I. ASPARAGINE AND GLUTAMINE FORMATION IN LUPINUS ANGUSTIFOLIUS, VICIA ATROPURPUREA, AND CUCURBITA PEPO

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The extensive literature on the amide metabolism of seedlings, and especially the classical work of Schulze and of his pupils, was thoroughly reviewed and discussed by Chibnall in 1939 (1). It has been established that during the early stages of growth, whether in darkness or in light, the seed protein undergoes digestion, presumably to amino acids, and that a greater or smaller proportion of the nitrogen of the amino acids is subsequently found, chiefly in the rapidly growing sprout, in the form of one or both of the amides asparagine and glutamine. At a later stage, with plants grown under normal conditions, these substances furnish nitrogen for the synthesis of proteins in the cells of the developing plant.

It was held by Schulze (2) that the first stage in the conversion of amino acids to amides is an oxidation reaction whereby ammonia is produced. Ammonia then combines with suitable non-nitrogenous compounds, probably derived from the carbohydrates, to give one or both of the amides. Reasons have been advanced by several investigators (1, 3, 4) for the assumption that the specific precursors of asparagine and glutamine are respectively oxalacetic and α-ketoglutaric acids which arise as intermediary products in carbohydrate metabolism. However, next to nothing has been established regarding the enzyme mechanisms whereby these various transformations are brought about in the plant, and the details of the chemical reactions and the sequence in which they occur are still subjects for speculation and debate.

In the present paper, data are presented to illustrate some of the chemical changes that take place when the seeds of three species of plants are allowed to sprout in darkness under conditions of high humidity. *Lupinus angustifolius*, a blue lupine, was selected as representative of a group of plants known to possess an unusually intense asparagine metabolism (5). This particular species of lupine has the additional advantage of being available from an American-grown crop.[1] *Vicia atropurpurea* (purple vetch) was

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[1] Seeds of *Lupinus angustifolius* grown in Florida, which had a germination rate of about 94 per cent, were obtained through the courtesy of Mr. C. W. Baker of F. H. Woodruff and Sons, Inc., Milford, Connecticut. The germination rate of this species appears to be seriously affected by improper conditions of storage.

likewise selected because of its availability in this country and also because it represents a species with a'moderately intense asparagine metabolism. Furthermore, it is one of the vetches the seeds of which germinate well without special treatment. The two varieties of *Cucurbita pepo* (summer squash) were selected as representative of species known to possess a moderately intense glutamine metabolism (6). Attention is drawn to the possibility of the practical application of the present results to the preparation both of asparagine and of glutamine, at least on the laboratory scale.

EXPERIMENTAL

Preparation of Etiolated Seedlings—The technique of growing the seedlings described by Vickery, Pucher, and Deuber (7) was modified to permit the removal of individual samples from time to time. The seeds were germinated in wire mesh baskets 22 cm. in diameter and 20 cm. deep, supported about 2 cm. above shallow pans so adjusted that about 1 cm. of water would accumulate in them. The baskets were arranged in two layers in a dark cabinet, and water was supplied through spray nozzles[2] centrally placed just above the tops of the baskets at each level. Each basket contained 125 gm. of seeds evenly spread on the bottom. All equipment must be scrupulously clean and should be sterilized before use.

Water at a temperature of 20–24° was supplied to the nozzles continuously until germination was well established, and thereafter during the night. Single baskets were removed at intervals and the seedlings, without discarding the testa tissue, were prepared for analysis by being rapidly dried in a ventilated oven in a current of air at 80° and subsequently ground to a fine powder in a Wiley mill.

Analytical Methods—*Ammonia* and the *amides* were determined as described in earlier papers from this laboratory (8, 9), asparagine and glutamine nitrogen being taken as twice the respective amide nitrogen. *Water-soluble nitrogen* was determined in hot water extracts of the dried tissue. The solutions were cooled and treated with an excess of trichloroacetic acid in order to remove soluble protein, and the precipitate was centrifuged off and thoroughly washed, nitrogen being determined in the combined solution and washings. *Protein nitrogen* was arbitrarily taken as the difference between the total nitrogen and the water-soluble nitrogen. The "crude dry weight" represents the weight of the dried, unground tissues, the small percentage of analytically determinable water present being ignored.

Results with Lupinus angustifolius—Table I shows the data obtained by the analysis of the etiolated seedlings of *Lupinus angustifolius*. The rapid loss of organic solids by respiration is well shown by the data for the dry

weight of the tissues, while the constancy of the total nitrogen during the first 12 days indicates that no significant volatilization of ammonia nor leaching of soluble nitrogenous components took place. However, the observations at 14 and 16 days show a marked loss of nitrogen from the seedlings. This correlates in time with the precipitous drop in asparagine content and an equally sudden rise in the quantity of ammonia present.

TABLE I

Composition of Seedlings of Lupinus angustifolius Sprouted in Darkness with Liberal Supply of Tap Water, but without Nutrient Salts

Figures not otherwise designated represent gm. per 100 gm. of seed.

Days	0	2	6	10	12	14	16
Fresh weight	100	319	722	1020	1120	1077	960
Crude dry weight	93	85.7	81.7	74.9	71.6	66.6	52.2
pH of extracts of dried tissue	5.64	5.49	5.13	5.08	5.23	5.82	6.42
Total N	5.00	5.12	5.03	4.95	4.84	4.22	2.64
Water-soluble N	1.03	1.42	2.96	3.78	3.86	3.18 (3.96)	1.88 (4.24)
Protein N	3.97	3.70	2.07	1.17	0.98	1.04	0.76
Ammonia N	0.023	0.021	0.027	0.047	0.089	0.32 (1.10)	0.53 (2.89)
Glutamine "	0.000	0.000	0.086	0.102	0.022	0.000	0.000
Asparagine "	0.04	0.29	1.30	1.96	2.20	1.64	0.31
Determined soluble N	0.063	0.311	1.413	2.109	2.311	1.96 (2.74)	0.84 (3.20)
Undetermined soluble N	0.97	1.11	1.55	1.67	1.55	1.22	1.04
Asparagine N, % soluble N	3.88	20.4	43.9	51.9	57.0	51.6 (41.4)	16.5 (7.3)
Glutamine N, % soluble N	0.00	0.00	2.9	2.7	0.6	0.0	0.0
Ammonia N, % soluble N	2.2	1.5	0.91	1.2	2.3	10.2 (27.8)	28.0 (68.1)

The tissues at 12 days were turgid and healthy and had accumulated a strikingly high proportion of asparagine, but 2 days later the plants had undergone an obvious internal collapse. Careful examination failed to reveal the presence of fungi or microorganisms[3] which could account for the sudden change, and it is therefore inferred that exhaustion of some tissue

[3] We are deeply indebted to Dr. J. G. Horsfall of the Department of Plant Pathology at this Station for these examinations.

component or components took place which resulted in an alteration in the general course of the metabolism. The phenomenon has not hitherto been encountered in this laboratory and no record of a similar behavior has been found in the literature.[4] For example, Merlis (5), working with the same species, but with seeds of European origin, was able to prolong the culture for 18 days with continuous increase in asparagine, and Lugg and Weller (11), working in Australia, record a high asparagine content after 14 days. Nevertheless repeated experiments showed that, with the samples of seeds of this species from both 1942 and 1943 crops that we have studied, more or less intense decomposition of asparagine invariably began after about 12 days of culture.

The seedlings harvested after 14 and 16 days smelled of ammonia and, before being dried, yielded a strongly alkaline water extract. The excess of ammonia disappeared during the drying, however, and only a moderate effect upon the pH of the water extracts of the dry tissue was apparent. The data in Table I for these two samples have been calculated from the direct analyses of the dried material. For certain items calculations have also been made on the assumption that the nitrogen lost represents ammonia volatilized during the drying operation. The figures thus obtained, enclosed in parentheses, doubtless represent a closer approximation to the composition of the plants than do the others.

The transformation of protein into water-soluble components and the rapid accumulation of asparagine are clearly illustrated. At the expiration of 12 days, three-quarters of the protein, as measured by the insoluble nitrogen, had disappeared and the equivalent of about 75 per cent of this nitrogen had been transformed into asparagine. The observations agree with those of Merlis fairly closely and are characteristic of the behavior of other species of lupine studied by Schulze, Prianischnikow, and others (see (1)).

The proportion of glutamine produced in this species is of a much smaller order of magnitude than that of asparagine.[5] The maximum concentration

[4] A partial analogy is to be found, however, in the behavior of the ammonia in tobacco leaves subjected to culture in darkness (10). Under these circumstances synthesis of asparagine proceeded at a relatively constant rate; the ammonia on the other hand remained at a low level for from 3 to 4 days and then suddenly began to accumulate much as in the present case, although there was no coincident effect upon the rate of asparagine synthesis.

[5] From the present data it is impossible to determine whether the glutamine was produced by a synthetic process analogous to that which gave rise to the asparagine, or whether the small amount of glutamine observed was liberated directly from the digested protein. The fact that glutamine was detectable only during the period when protein hydrolysis was proceeding at a maximum rate suggests, however, that the second alternative must be seriously considered. In this connection see Chibnall ((1) pp. 58–62).

was reached at 8 days (not shown in Table I) and amounted to only 3.1 per cent of the soluble nitrogen. Subsequently the glutamine diminished, and at 12 days only a trace remained; none could be detected in the 14 and 16 day samples.

If Schulze's hypothesis with respect to the intermediary function of ammonia be granted, the synthesis of asparagine clearly took place with high efficiency, since only a small amount of ammonia had accumulated as such even at the end of 12 days. It would seem obvious, however, that, as soon as active asparagine synthesis ceased, ammonia increased rapidly at the

TABLE II

Composition of Seedlings of Vicia atropurpurea Sprouted in Darkness with Liberal Supply of Tap Water, but without Nutrient Salts

Figures not otherwise designated represent gm. per 100 gm. of seed.

Days	0	4	9	16	19	22	26
Fresh weight	100	386	609	830	972	994	1048
Crude dry weight	95.0	84.8	79.8	71.0	67.0	65.0	59.4
Total N	4.23	4.20	4.38	4.12	4.17	4.09	3.80
Water-soluble N	0.82	1.22	2.28	2.68	2.75	2.74	2.61
Protein N	3.41	2.98	2.10	1.44	1.42	1.35	1.19
Ammonia N	0.013	0.004	0.008	0.015	0.025	0.061	0.097
Glutamine "	0.086	0.118	0.140	0.150	0.200	0.176	0.112
Asparagine "	0.108	0.074	0.512	1.05	1.09	1.07	1.02
Determined soluble N	0.207	0.196	0.660	1.22	1.32	1.30	1.23
Undetermined soluble N	0.61	1.02	1.62	1.46	1.43	1.44	1.38
Asparagine N, % soluble N	13.2	6.06	22.5	39.3	39.9	38.9	39.2
Glutamine N, % soluble N	10.5	9.7	6.1	5.6	7.3	6.4	4.3
Ammonia N, % soluble N	1.6	0.33	0.35	0.56	0.91	2.23	3.71

expense of the asparagine already present. The general picture is that of a series of interconvertible substances in a quantitatively steady state with respect to one another, the apparent equilibrium point being disturbed after the expiration of 12 days by the exhaustion of some component essential to the continued synthesis of asparagine. It is hoped that more detailed chemical examination will throw some light on the nature of this component.

Results with Vicia atropurpurea—Table II shows data obtained with seedlings of the purple vetch. As in the lupine experiment, the steady loss of organic solids through respiration is well shown, but the data for

total nitrogen are somewhat less satisfactorily constant, presumably owing to variation between individual samples. Because of this it cannot be certain that loss of nitrogen occurred during the drying of the 26 day sample, although this seems probable. The ammonia nitrogen at no time approached the concentration noted in lupine seedlings of the same age, and was low even at the end of the experiment.

Vetch seedlings in general produce smaller relative proportions of asparagine than lupines (12) under otherwise similar conditions. From the metabolic point of view, the most significant feature of the present data is that the asparagine content of the tissues reached a maximum in from 16 to 19 days, which was maintained at a nearly constant level until the end. The contrast with the blue lupine seedlings is marked, and the behavior likewise differs from that of white lupine seedlings (7) in which asparagine accumulation continued, though at a gradually diminishing rate, for 30 days. Furthermore this type of behavior is not necessarily characteristic of vetch species in general, since unpublished data obtained in this laboratory by Dr. C. G. Deuber indicate that, in seedlings of *Vicia villosa*, asparagine synthesis continued at an almost undiminished rate until the termination of the experiment at 20 days. Accordingly, generalization with respect to the rate and extent of asparagine synthesis in seedlings of different plant species is scarcely justified at the present time.

The course of the behavior of the glutamine in the purple vetch seedlings likewise differs from that in the blue lupines. There was an appreciable quantity in the original seeds of a substance which responded to the analytical method for glutamine, and this slowly increased by approximately 100 per cent of itself, reaching a maximum at 19 days and thereafter decreasing. There is little suggestion of the occurrence, as was noted with the blue lupines, of an intense oxidation which destroyed all of the glutamine and likewise attacked the asparagine, although the gradual accumulation of ammonia towards the end of the culture period may be interpreted as an indication that oxidation reactions were beginning to assume dominance.

Results with Cucurbita pepo—Table III shows the data from two experiments with different varieties of *Cucurbita pepo* seedlings. The general picture of loss of organic solids and protein and increase in amides and ammonia is similar to that for the lupine and vetch species shown in Tables I and II. The rate of disappearance of the protein was less than in the case of the lupines and resembled that of the vetches. However, synthesis of amides took place upon a definitely smaller scale. Whereas in the vetch seedlings the equivalent of somewhat more than 60 per cent of that part of the protein nitrogen which underwent digestion was ultimately converted to asparagine, in the squash seedlings the equivalent of only from a quarter to one-third of the nitrogen of the digested protein was found in the form of glutamine and asparagine.

The most striking difference is, however, in the relative proportions of the two amides produced. In the squash plants, glutamine was present at the end in a concentration more than double that of the asparagine. At first glance it would appear that the evidence for a synthetic process whereby glutamine is produced is conclusive, and for the case of the *Ricinus communis* seedlings considered by Chibnall ((1) p. 61), in which the relative proportion of glutamine present at the end of the culture was considerably higher than in the present case, there is indeed little doubt. However, at the end of 21 days of culture of the squash seedlings, some 3.3 gm.

TABLE III

Composition of Seedlings of Two Varieties of Cucurbita pepo Sprouted in Darkness with Liberal Supply of Tap Water, but without Nutrient Salts

Figures not otherwise designated represent gm. per 100 gm. of seed.

Variety	Connecticut straight neck			Prolific		
Days	0	12	19	0	16	21
Fresh weight	100	1294	1620	100	1192	1530
Crude dry weight	95.8	83.4	75.2	95.4	78.8	75.0
Total N	5.25	4.79	4.76	5.66	5.12	5.25
Water-soluble N	0.41	1.84	2.60	0.46	1.99	2.66
Protein N	4.84	2.95	2.16	5.20	3.13	2.59
Ammonia N	0.002	0.067	0.184	0.003	0.069	0.100
Glutamine "	0.012	0.228	0.498	0.006	0.320	0.642
Asparagine "	0.040	0.116	0.242	0.042	0.126	0.262
Determined soluble N	0.054	0.461	0.924	0.051	0.515	1.00
Undetermined soluble N	0.36	1.38	1.68	0.41	1.47	1.66
Asparagine N, % soluble N	9.86	9.0	9.3	9.1	6.3	9.8
Glutamine N, % soluble N	2.9	12.4	19.2	1.4	16.1	24.1
Ammonia N, % soluble N	0.5	3.6	7.1	0.6	3.5	3.7

of glutamine had been formed at the expense of about 16 gm. of protein, an apparent yield of 20 per cent. Osborne and Clapp (13) were able to isolate only 12.4 per cent of glutamic acid from the globulin of an allied species of squash, but there is ample reason today (14) to assume that these early analytical values may have been considerably too low, and it must further be remembered that the crystallizable globulin does not represent the entire protein content of the seeds. Thus the present experiment does not provide completely unequivocal evidence for glutamine synthesis. On the other hand, if all of the glutamine present arose directly from the protein, not only must it be assumed that the proteins of these seeds are unusually rich in glutamic acid combined in the protein as the respective

amide, but it is further implied that the glutamine, once liberated, played no further rôle in the metabolic processes. This is contrary to the evidence provided by the lupine experiment for the presence in seedling tissues of oxidative mechanisms capable of destroying both glutamine and asparagine. The weight of evidence is thus in favor of the view that a part at least of the glutamine in the squash seedlings arose as the result of synthetic processes in which ammonia derived from the oxidation of protein decomposition products played a part. This interpretation is in line with inferences drawn from the case of glutamine synthesis in beet root tissue from plants subjected to culture in the presence of ammonium salts (15).

The present experiments with squash seedlings suggest that this organism may have interest in connection with the laboratory preparation of glutamine. The concentration of glutamine in terms of dry weight of the tissues of the seedlings cultured for 21 days was 4.4 per cent. Unfortunately, however, isolation in pure form involves separation by fractional

TABLE IV

Preparation of Asparagine from Seedlings of Lupinus angustifolius

Weight of seeds	Age of seedlings	Fresh weight	Asparagine hydrate per 100 gm. seeds		Purity of asparagine nitrogen analysis
			By analysis	By isolation	
gm.	*days*	*kg.*	*gm.*	*gm.*	*per cent*
1651	12	18.4	10.8	8.7	99.6
2000	15	21.1	3.9	2.8	93.5

crystallization from the asparagine likewise present, as well as from a complex mixture of other substances. Although this is by no means impossible, an instance in which it was attempted (16) suggests that the conditions under which the crystallization is carried out will require careful study before satisfactory yields may be expected.

Preparation of Asparagine from Lupinus angustifolius Seedlings—In order to confirm the impression gained from the experiment the data of which are shown in Table I, two larger scale cultures were carried out with the object of testing the usefulness of *L. angustifolius* seedlings for asparagine preparation on the laboratory scale. In one of these, tissue from 1651 gm. of seeds was harvested at 12 days, when maximum concentration of asparagine was anticipated. In the second, in which 2000 gm. of seed were used, the culture time was extended to 15 days in order to study the effect of the spontaneous decomposition of the asparagine. The essential data from these experiments are shown in Table IV; the technique employed was identical with that recently described (7) save that the crops of crude asparagine were washed with ice-cold 25 per cent alcohol instead of with ice

water. The figure for the yield from the first experiment includes only the first crop material after one recrystallization. An additional 1.2 per cent was secured from the mother liquors after concentration or, in all, 91.6 per cent of the asparagine indicated by indirect analysis of the extract. The yield of pure material was thus somewhat less than that previously obtained from *Lupinus albus* seeds (7), but is materially greater than was obtained from soy beans, the only seed of American origin other than *L. angustifolius* that has been studied in this connection. Furthermore, since only 12 days of culture time are required as against 20 days for a similar yield from white lupine seedlings, there are manifest advantages in using *L. angustifolius* seeds for asparagine preparation.

The result of the second experiment illustrates the failure that must be anticipated if culture of this species is allowed to proceed too long. Not only was the yield depressed so much as to make isolation hardly worth while, but the asparagine obtained was impure and it was found impossible effectively to improve its quality by further recrystallization or even by treatment in solution with lead acetate.

Preparation of Aspartic Acid from Asparagine

Asparagine derived from etiolated seedlings provides by far the most readily available source of aspartic acid. The only method described in the literature which appears to give good yields is that suggested by Schiff (17). A brief examination of his procedure has accordingly been made.

The method consists essentially in hydrolysis of asparagine in the presence of 2 moles of hydrochloric acid; 1 mole of ammonium hydroxide is then added and the aspartic acid is allowed to crystallize in the cold. Schiff observed that the concentration of ammonium chloride in the aqueous solution from which the aspartic acid is separated must not exceed 12 per cent if a high yield is to be obtained. In the present work it has been found that the yield is improved by the addition of alcohol and also by readjustment of the reaction after this addition to the approximate isoelectric point of aspartic acid (pH 3.0). Strict adherence to details has been found essential to obtain reproducible results.

As an example, the following may be given: 20 gm. of asparagine hydrate were dissolved in 78 ml. of 3.5 N hydrochloric acid (a slight excess over 2 moles) and the solution was boiled under a reflux condenser for 3 hours, cooled, and 39 ml. of 3.5 N ammonium hydroxide, followed by 2 volumes of 95 per cent alcohol, were added. To the suspension of aspartic acid which precipitated at once, a few drops of bromophenol blue indicator solution were added and then 3.5 N hydrochloric acid drop by drop until the color changed to yellow; from 1.5 to 2.5 ml. are ordinarily required. After being chilled overnight, the solution was filtered, the aspartic acid was washed

with cold 50 per cent alcohol until a few drops of wash fluid gave only a trace of color with Nessler's solution, and the crystals were dried at 105°. The small quantity of ammonium salt that still remains can be removed by recrystallization from boiling water in which the acid is soluble to the extent of nearly 7 per cent, whereas the solubility at 0° is about 0.2 per cent (18).

Table V shows the data from a series of experiments of which the first illustrates Schiff's original procedure, the second the effect of the addition of 1 volume of alcohol, and the remainder the effect of the addition of 2 volumes of alcohol followed by adjustment of the reaction.

For these last experiments, asparagine of 95.5 per cent initial purity obtained as second crop material from seedling tissue extracts was employed. The corrected yield figures are calculated upon the assumption that the contaminant in this impure asparagine was non-nitrogenous. The constancy of the composition of the aspartic acid (theory, 10.52 per cent of

TABLE V

Preparation of Aspartic Acid from Asparagine by Schiff's Method

Conditions for separation of aspartic acid	Aspartic acid as isolated			Aspartic acid corrected for ammonium chloride	
	Yield	Ammonium chloride	N content	Yield	Calculated N content
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
From water	90.3	0.36	10.62	89.9	10.52
" " + 1 volume alcohol	93.3	0.30	10.59	92.9	10.51
" " + 2 volumes "	99.4	0.19	10.57	99.2	10.52
with adjustment of pH	101.3	1.00	10.74	100.3	10.47
	99.2	0.34	10.56	98.9	10.47
	100.4	0.40	10.61	100.0	10.50

nitrogen), after correction for its separately determined ammonium chloride content, is evidence that the impurity was satisfactorily removed.

SUMMARY

Seedlings of *Lupinus angustifolius*, after having been sprouted in darkness without nutrient salts for 12 days, accumulated asparagine in the tissues to the extent of about 11 per cent of the original weight of the seeds. The asparagine content thereafter dropped rapidly to a low level with the simultaneous production of ammonia. Invasion of the young plants by microorganisms could not be demonstrated, and the observations are therefore interpreted as evidence for a suddenly initiated alteration in the course of the metabolism owing to the exhaustion of non-nitrogenous components essential for the synthesis of asparagine. Nevertheless it is shown that the American-grown variety of this species can be satisfactorily used for the laboratory preparation of asparagine.

Seedlings of *Vicia atropurpurea*, under similar conditions, reached a maximal content of asparagine in from 16 to 19 days, which was maintained with little further change for 7 days longer. At the highest, however, the yield was only a little more than 5 per cent of the original weight of the seeds. This species is therefore less satisfactory for asparagine preparation than the blue lupine. Glutamine was present in the seedlings of both of these species but only in negligible quantities.

Seedlings of *Cucurbita pepo* behave quite differently from those of the lupine and vetch in that glutamine is produced to the extent of somewhat more than 3 per cent of the original weight of the seed in 21 days. Asparagine synthesis likewise takes place but in an amount only about half that of the glutamine. The use of this readily available material for the preparation of glutamine on a laboratory scale is therefore an inviting possibility.

The details of Schiff's procedure for the preparation of aspartic acid from asparagine have been studied and conditions which give essentially quantitative yields are described.

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THE EFFECT OF ANOXIA ON THE METABOLISM OF LIVER SLICES FROM FED AND FASTED RATS*

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With regard to the oxygen uptake of slices of liver, Beecher and Craig (2) found no difference between cats in profound hemorrhagic shock and controls. In rats in hemorrhagic shock that had been fasted for 24 hours, Engel, Winton, and Long (3) observed changes in blood chemistry and unpublished work in their laboratory showed a sharp depression in oxygen uptake by surviving liver slices. They reported these results as evidence of a decrease in hepatic function resulting from anoxia. In these two studies on liver slices from animals in shock the cats had been fed on a high protein diet, the rats on a high carbohydrate diet. Mirsky, Rosenbaum, Stein, and Wertheimer (6) and Newburger and Brown (7) found that the liver glycogen of rats on a high carbohydrate diet was minimal after 24 hours of fasting, whereas there was little decrease in liver glycogen in fasting when the previous diet had been high in protein. The difference between our results and those obtained in Dr. Long's laboratory need not necessarily be due to diet. Nevertheless, the effect of a 24 hour fast on the ability of the metabolism of liver slices to recover from anoxia seemed worth investigating briefly. The liver tissue was subjected to anoxia *in vitro*; thus any complicating factors associated with shock were avoided.

Method

Adult male and female rats of Wistar strain were employed. Previously they had been used for breeding purposes for some time and discarded. The diet, Purina dog chow, had a 67 per cent carbohydrate content. Fourteen rats were divided at random into two groups of seven. One group had access to food until sacrificed. From the second group, food was withheld for 24 hours. After the rats had been sacrificed, the livers from both groups were treated in the same way, as follows: Slices were cut into Ringer-phosphate-glucose medium at about 25°. The medium was made up to contain NaCl 0.118 M, KCl 0.0024 M, CaCl$_2$ 0.0017 M, MgCl$_2$ 0.00066

M, NaH_2PO_4 0.003 M, Na_2HPO_4 0.017 M, and glucose 2 mg. per cc., at pH 7.4. Slices from each liver were distributed among three conical Warburg vessels containing 2.0 cc. of medium and 0.1 cc. of 20 per cent NaOH in the inset. About 20 mg. (final dry weight) of tissue were placed in each vessel. Within 15 minutes after sacrifice the vessels were placed in a bath at 38° and shaken at 120 cycles per minute. Vessel 1 was filled with oxygen, and Vessels 2 and 3 with nitrogen containing 0.5 per cent oxygen; gas was passed through the vessels for 5 minutes. The zero time reading was taken 10 minutes later for the manometric determination of oxygen uptake by the first method of Warburg (8). Successive readings were taken at 15 minute intervals. Immediately after the reading at 15 minutes the nitrogen in Vessel 2 was replaced with oxygen and readings were con-

TABLE I

Oxygen Uptake by Liver Slices from Fed and Fasted Rats

Means and standard errors of means; $n = 7$.

Time under nitrogen	Time interval	Oxygen uptake	
		Fed	Fasted
min.	*min.*	*c.mm. per mg. per hr.*	*c.mm. per mg. per hr.*
0	0– 60	7.3 ± 0.34	8.2 ± 0.15
	75–135	7.4 ± 0.36	8.0 ± 0.17
	135–195	7.3 ± 0.32	7.7 ± 0.15
25	30– 75	6.5 ± 0.30	5.7 ± 0.22
	75–135	6.8 ± 0.22	5.3 ± 0.08
	135–195	6.4 ± 0.28	4.3 ± 0.21
70	75–135	5.7 ± 0.20	2.3 ± 0.48
	135–195	5.2 ± 0.21	1.7 ± 0.28

tinued. The time under nitrogen for Vessel 2 was 25 minutes. Immediately after the reading at 60 minutes the nitrogen in Vessel 3 was replaced with oxygen and readings continued. The time under nitrogen for Vessel 3 was 70 minutes. At 195 minutes all three vessels were removed from the bath; the liver slices were removed and dried to constant weight. The lactic acid content of the medium was determined by the method of Barker and Summerson (1).

Results

The data for oxygen uptake are recorded in Table I. In the liver slices from both fed and fasted rats the rate of oxygen uptake was nearly constant over a 3 hour period. Fasting appeared to have little if any effect on the rate of oxygen uptake when the oxygen supply was maintained in the usual way (Vessel 1). In tissue from fed animals, exposures to nitro-

gen of 25 and 70 minutes were followed by decreases in oxygen uptake of about 10 and 25 per cent respectively. In tissue from fasted animals the decreases amounted to about 35 and 75 per cent respectively.

Little lactic acid was formed in the presence of oxygen. During or after exposure to nitrogen for 70 minutes about twice as much was formed by fed livers as by fasted ones (Table II).

TABLE II

Lactic Acid Output

Means and standard errors of means; $n = 6$.

Time under nitrogen	Total lactic acid recovered		Increment due to exposure to nitrogen	
	Fed	Fasted	Fed	Fasted
min.	*mg. per gm.*	*mg. per gm.*	*mg. per gm.*	*mg. per gm.*
0	8 ± 1.0	1 ± 0.3		
25	15 ± 1.6	3 ± 0.7	7 ± 1.2	2 ± 0.6
70	22 ± 1.9	8 ± 0.9	15 ± 1.2	8 ± 1.0

DISCUSSION

The Q_{O_2} obtained in these experiments is lower than one would ordinarily expect for rat liver. A Q_{O_2} of 7.3 has been reported by Kleiber (5). In comparing his results with those of Field, Belding, and Martin (4) Kleiber attributed the low value to the fact that his rats were somewhat larger than theirs. The rats used in the present experiments were large and full grown, but the age and weight were not recorded.

In so far as hepatic function may depend on the metabolic processes studied here, the decline in rate of oxygen uptake following exposure to nitrogen supports the conclusion of Engel, Winton, and Long (3) regarding the sensitivity of the liver to anoxia under the conditions of their experiments. The amplification of the decline after nitrogen in the tissue from fasted rats, however, may raise the question of the importance of the fast and the nature of the previous diet in contributing to the changes in surviving metabolism in liver from animals in hemorrhagic shock. Whether the glycogen content or some other factor is responsible for the greater sensitivity of fasted liver tissue to anoxia *in vitro* remains undetermined.

SUMMARY

Measurements were made of oxygen uptake and lactic acid output by liver slices from rats fed on a high carbohydrate diet. The oxygen uptake was depressed by 25 per cent following 70 minutes of exposure to nitrogen. When the rats had fasted for 24 hours previously, the depression after nitrogen was 75 per cent. Fasting also diminished the lactic acid output.

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GLYCERALDEHYDE 1,3-DIPHOSPHATE (DIMERIC)*

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WITH A SECTION IN THE TEXT BY OTTO MEYERHOF

Warburg and Christian (1) have purified and isolated the ferment protein which, in the presence of phosphate and pyridine nucleotide, catalyzes the oxidation of triose phosphate to glyceric acid diphosphate (R-diphosphoglyceric acid). The use of the purified ferment protein enabled Negelein and Brömel (2) to isolate the primary oxidation product of phosphotriose, the 1,3-diphosphoglyceric acid, in the form of its strychnine salt. The phosphate taken up in the oxidation process reappears in the carboxylic group.[1]

In an earlier paper Negelein and Brömel (4) expressed the view that the *d* form of glyceraldehyde 3-phosphoric acid (Fischer-Baer ester) (5) reacts with inorganic phosphate, forming a *d*-glyceraldehyde diphosphate. Being the precursor of glyceric acid 1,3-diphosphate, its constitution, if it exists as a primary valence compound, may be expressed by formula (I) or (II). Negelein and Brömel leave the question undecided as to whether such a diphosphate of glyceraldehyde would exist in a (hydrated) monomeric form (I) or as a dimeride (II).
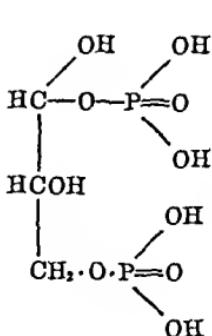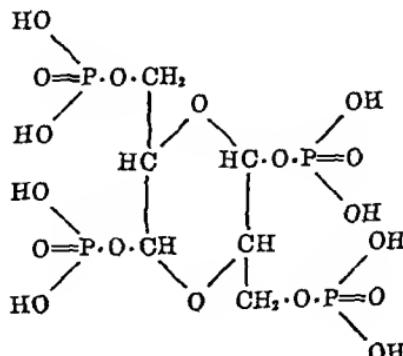
(I)   (II)

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* With regard to the nomenclature see the second paragraph before the section "Experimental."

[1] In the American literature the significance of these observations is comprehensively discussed by Meyerhof (3).

Obviously formula (I) represents a compound of a new type, which, even if it could be synthesized, would be difficult to isolate owing to the great lability which may be expected of such an aldehyde-hydrate phosphate.[2]

The synthesis of compound (II), however, was possible. Therefore it seemed advisable to undertake its preparation and base the decision whether the glyceraldehyde diphosphate which is assumed to occur in nature exists in its monomeric or dimeric form upon the results of the biological investigation of the dimeric diphosphate.

In order to synthesize the dimeric diphosphate of glyceraldehyde (8) we treated crystalline dimeric dl-glyceraldehyde with pyridine and diphenylphosphoryl chloride using the method of Brigl and Müller (9). We obtained the octaphenyl ester of compound (II) in yields up to 48 per cent (m.p. 110–111°). Molecular weight determinations, by several methods, gave approximately the expected molecular weight of the dimeric phenyl ester.

The phenyl groups of the octaphenyl ester were eliminated by catalytic hydrogenolysis in methanol with platinum and hydrogen at room temperature, yielding the free phosphate (substance (II)). Its crystalline acid barium and acid calcium salts were prepared.

Since pyridine, used in the preparation of the octaphenyl ester, had been shown to bring about the rearrangement of glyceraldehyde (10), it was necessary to demonstrate that the crystalline octaphenyl ester obtained was not a derivative of dihydroxyacetone. Biological and chemical experiments indicated that a partial hydrolysis of the dimeric glyceraldehyde 1,3-diphosphate produced glyceraldehyde 3-phosphate, thus establishing the new phosphate as a derivative of glyceraldehyde.[3]

The molecular weight of the phenyl ester shows that the ester and the free phosphate are derivatives of a dimeric form of glyceraldehyde. Recently, Summerbell and Rochen (12), in confirmation of the results of Bergmann and Miekeley (13), have proved convincingly that glycolic aldehyde, the lower homologue of glyceraldehyde, is a dioxane derivative. In analogy herewith, the dimeric glyceraldehyde was formulated as a dioxane derivative. The position of the four hydroxyls in the dimeric glyceraldehyde has been proved previously, as depicted in formula (II) (14).

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[2] A similar additive compound between phosphoric acid and the keto group of pyruvic acid is postulated by Lipmann (6). Cf. also the recent publications of Baer (7) pertaining to the existence of analogous "additive compounds" between α-keto acids or α-keto alcohols with water, alcohols, and probably also phosphoric acid. The formation of these compounds has been found to be indispensable for the oxidative cleavage (dehydrogenation) of the α-keto alcohols and α-keto acids with lead tetraacetate.

[3] Cf. also the paper immediately following, by Baer and Fischer (11), on the simplified preparation of dl-glyceraldehyde 3-phosphate.

For all these reasons, formula (II) was ascribed to the new synthetic phosphate.

The dimeric diphosphate is easily hydrolyzed by dilute mineral acids. There seems to be a considerable difference in the ease of hydrolysis of the two types of phosphoric esters present in this compound. Whereas a short acid hydrolysis of the dimeric diphosphate forms glyceraldehyde 3-phosphate (Meyerhof), a prolonged hydrolysis yields methylglyoxal as the final product. Against the action of alkali, the substance is remarkably stable. A hydrolysis by phosphatases from dog feces at pH 9.6 is described.

The dimeric diphosphate was tested for its biological activity by Meyerhof and Drabkin. They very kindly placed their results at our disposal for publication. A preliminary note by Professor O. Meyerhof appeared in this *Journal* (15). All biological tests so far have been negative, thereby excluding the dimeric formula (II) from representing the constitution of the hypothetical intermediate. This leaves formula (I) as a possibility.

This conclusion may only be valid if our new diphosphate is an ordinary racemic mixture of dimeric *dd*-glyceraldehyde 1,3-diphosphate and its enantiomorph, dimeric *ll*-glyceraldehyde 1,3-diphosphate. If in the dioxane ring of our compound, *d*-glyceraldehyde diphosphate and *l*-glyceraldehyde diphosphate are firmly bound together so that they cannot separately be attacked by the enzyme without opening the ring, there is a slight possibility that the dimeric *dd*-glyceraldehyde diphosphate is still the expected intermediate.

The microanalyses were carried out in this laboratory by Miss Shirley Platt.

EXPERIMENTAL

Glyceraldehyde 1,3-Bisdiphenylphosphate (Dimeric)

To 7.5 gm. of ice-cold dry pyridine were added with vigorous stirring 22.0 gm. of ice-cold diphenylphosphoryl chloride[4] (9), followed immediately by the addition of 3.6 gm. of finely powdered and dry racemic glyceraldehyde.[5] During the first 2 hours of the phosphorylation the outside temperature was kept at about 10–15°. At the end of 2 hours the stirring was discontinued and the pasty mixture allowed to stand at room temperature for an additional 24 hours. In order to isolate the phenyl ester from the strongly colored and completely solidified reaction product, it was stirred

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[4] Prepared from phenol of high purity.

[5] The glyceraldehyde, recrystallized once from water, was purified by refluxing the substance with 8 times its weight of dry acetone over a period of 2 hours. The aldehyde was sucked off, finely ground, and passed through a 200 mesh sieve. M.p. 141–142°.

up with 50 cc. of ice-cold 95 per cent ethanol into a homogeneous paste and filtered with suction. The residue was washed on the filter with a minimum of ice-cold ethanol until colorless. The crude product was dissolved in 25 cc. of lukewarm dioxane and freed from unchanged glyceraldehyde (about 0.7 gm.). The phenyl ester was precipitated from the filtrate by adding, in small portions, 150 cc. of petroleum ether (b.p. 80–100°). 7 gm. (39.3 per cent)[6] of pure octaphenyl ester (m.p. 110–111°) were obtained.

The substance is readily soluble in cold dioxane, bromoform, and chloroform, and in warm ethyl acetate, benzene, and carbon tetrachloride, but only slightly soluble in boiling petroleum ether, and insoluble in water.

$C_{54}H_{48}O_{18}P_4$ (1108.5). Calculated. C 58.45, H 4.36, P 11.20
Found.   " 58.22, " 4.57, " 11.50

Determination of Glyceraldehyde—When a solution of 1 gm. of 2,4-dinitrophenylhydrazine in 60 cc. of 2 N hydrochloric acid was heated for 1 hour on a boiling water bath with 0.567 gm. of phenyl ester there was obtained 0.437 gm. (98.9 per cent) of methylglyoxal 2,4-dinitrophenylosazone. Under these conditions the triose monophosphates similarly yield methylglyoxal 2,4-dinitrophenylosazone quantitatively.

The molecular weight was determined cryoscopically. Solvent 72.25 gm. (25.0 cc.) of bromoform; 1.7522 gm. of phenyl ester; depression 0.376°.

Mol. wt. Calculated, 1108.5; found, 929

Glyceraldehyde 1,3-Diphosphate (Dimeric) (II)

Reductive Cleavage—In order to obtain the free acid (II) a suspension of 4.4 gm. of phenyl ester and 2.0 gm. of platinum oxide (Adams' catalyst) in 100 cc. of dry methanol at room temperature was vigorously shaken in an atmosphere of hydrogen at a pressure of about 60 cm. of water. At the beginning of the reduction occasional cooling was applied to prevent alcoholysis. The reductive cleavage was complete in about 4 hours, at the end of which time the rate of absorption had become negligible (10 cc. per 15 minutes) and slightly more than the theoretical amount (32 moles) of hydrogen had been absorbed. With smaller amounts of ester, *viz.* 2.2 gm. or less, the time needed was 1 hour or less. The resulting solution of the free acid was concentrated *in vacuo* (bath temperature 25°) to a thick syrup.

Preparation of Acid Barium and Calcium Salts—The syrup obtained above was dissolved in 25 cc. of water; the solution was filtered into a crystallization dish containing a solution of 4.4 gm. of barium chloride in 25 cc. of water. The precipitation of crystallized acid barium salt was induced

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[6] Based on unrecovered glyceraldehyde. In a few experiments up to 48 per cent of the theoretical yield was obtained.

by gradual addition in small portions of 110 cc. of 95 per cent ethanol and completed by allowing the mixture to stand for 20 hours in a vacuum desiccator at 140 mm. of Hg above an open dish containing ethanol. The acid barium salt which was washed on the filter successively with 50 per cent ethanol, 95 per cent ethanol, and ether was dried in a high vacuum at room temperature. The yields of crude acid barium salt obtained in several experiments ranged from 2.23 to 2.87 gm. (72.7 to 93.5 per cent). In order to purify the acid barium salt and to obtain it in well crystallized form, the salt was reprecipitated from an acid solution as follows: a filtered solution of 2.3 gm. of crude barium salt in 30 cc. of 1 N hydrochloric acid was diluted by adding dropwise 25 cc. of 95 per cent ethanol over a period of 5 hours. The solution was kept for 20 hours in a vacuum desiccator (about 140 mm.) in the presence of alcohol, as described above. The barium salt obtained was washed with ethanol, followed by ether, and dried in a high vacuum at room temperature. About 50 per cent by weight of the crude salt was obtained in pure state. The following analyses indicate the presence of 2 moles of water of crystallization.

$C_6H_{12}O_{11}P_4Ba_2$ (770.8). Calculated. C 9.33, H 1.56, P 16.08, Ba 35.6
$C_6H_{12}O_{11}P_4Ba_2 + 2H_2O$ (806.8). Calculated. C 8.93, H 2.00, P 15.40, Ba 34.0
Found. " 8.82, " 2.01, " 15.4, " 32.8
" 8.67, " 2.17

The glyceraldehyde content of the acid barium salt was determined as described for the phenyl ester. 0.2352 gm. of barium salt in hot hydrochloric acid solution in the presence of an excess of 2,4-dinitrophenylhydrazine yielded 0.2385 gm. of methylglyoxal 2,4-dinitrophenylosazone (94.7 per cent of the theoretical, calculated for the acid barium salt + $2H_2O$).

The corresponding acid calcium salt was prepared from the free acid (syrup) as described for the barium salt.

$C_6H_{12}O_{13}P_4Ca_2$ (576.3). Calculated. C 12.50, H 2.08, P 21.55, Ca 13.9
$C_6H_{12}O_{13}P_4Ca_2 + 2H_2O$ (612.3). Calculated. C 11.75, H 2.61, P 20.3, Ca 13.1
Found. " 11.57, " 2.7, " 19.4, " 12.7

The neutral barium and calcium salts, which were also prepared, could be obtained only in amorphous state. The acid salts, as well as the neutral salts, are stable substances and in contrast to the dl-glyceraldehyde 3-phosphoric acid calcium salt do not form methylglyoxal spontaneously on standing at room temperature . All these salts, however, on heating in 2 N hydrochloric acid with an excess of 2,4-dinitrophenylhydrazine yielded almost quantitatively the 2,4-dinitrophenylosazone of methylglyoxal. The diphosphate is further distinguished by its stability towards alkali.

The new phosphate is readily hydrolyzed by phosphatase in alkaline solution.

Enzymatic Hydrolysis

Substrate-Buffer Solution—This is prepared by triturating 62.8 mg. of acid barium salt ($+2H_2O$) in 20.0 cc. of water with a slight excess of sodium sulfate. The filtrate was neutralized, diluted with 50.0 cc. of veronal buffer (pH 9.6), and made up with distilled water to a volume of 100.0 cc. The solution contained 8.82 mg. of phosphorus in 100.0 cc. (P determined colorimetrically).

Enzyme Solution—Using directions given by Armstrong (16) we isolated a highly active phosphatase from dog feces. The enzyme solution was prepared by dissolving the solid material in physiological saline to produce a concentration of approximately 50 units of phosphatase per 100 cc.

Hydrolysis—25.0 of the substrate-buffer solution were brought to 39.2° and 3.0 cc. of enzyme solution were added. Aliquots were removed at intervals of 5 minutes and the liberated phosphoric acid was determined colorimetrically. The results obtained are reported in Table I.

TABLE I

Enzymatic Liberation of Phosphoric Acid in Substrate-Buffer Solution

Time	Inorganic P in 100.0 cc.	Hydrolysis
min.	*mg.*	*per cent*
5	1.23	14
10	2.12	24
15	3.09	35
20	3.53	40
30	4.50	51
45	5.74	65
60	6.18	70
120	7.76	88

Experiments on Biological Activity

By OTTO MEYERHOF

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The dimeric glyceraldehyde 1,3-diphosphate of Baer and Fischer was tested for biological activity (*a*) directly, (*b*) after partial acid hydrolysis, and (*c*) after incubation with alkali. The main experiments consisted of the examination of the following three known reactions by substituting the new substance for the *d*-glyceraldehyde phosphate.

(A) *d*-Glyceraldehyde phosphate (or new substance) + cozymase + phosphate $\rightleftharpoons$
1,3-diphosphoglyceric acid + dihydrocozymase

(B) *d*-Glyceraldehyde phosphate (or new substance) + cozymase + arsenate $\rightarrow$
phosphoglyceric acid + dihydrocozymase + arsenate

(C) *d*-Glyceraldehyde phosphate (or new substance) $\rightleftharpoons$ dihydroxyacetone phosphate
$\Updownarrow$
hexose diphosphate

Reaction A was followed in the presence of the pure oxidizing enzyme of Warburg and Christian (1) by measuring the height of the absorption band at 340 $\mu\mu$ of dihydrocozymase by means of the photoelectric monochromator of Dr. Drabkin in this department.[7]

Reaction B was measured either in the same way or by manometric technique, since two acid groups develop in the reaction.

Reaction C was tested by phosphate determinations, since the phosphate group in the monophosphotrioses is alkali-labile; those of hexose diphosphate and those of the new substance are alkali-stable.

All these tests were *negative* for the new ester, *directly* or after *alkali* treatment. After appropriate partial *acid* hydrolysis a substance was formed which behaved like glyceraldehyde 3-phosphate (Fischer-Baer ester) in Reactions A to C as well as in the chemical tests of alkali lability, formation of methylglyoxal, and iodine titration.

TABLE II

Hydrolysis of New Ester in 0.02 N HCl at 100° in Solution Containing 0.663 Mg. of Organic P per Cc.

Time	Phosphorus	$k \times 10^{-3}$ (log₁₀)
min.	mg.	
2	0.217	85
4	0.282	35
10	0.360	16.6
15	0.387	8.5 ⎫
30	0.460	8.8 ⎬ 8.0
60	0.552	8.7 ⎭
180	0.657	

Acid Hydrolysis of New Ester—The procedure for testing the new ester in Reactions A to C after partial hydrolysis was based on the observation that at 100° in 0.02 to 0.1 N HCl the first half of the phosphate is split off very rapidly. During the first 2 minutes the velocity constant (k) for the hydrolysis in 0.02 N HCl is about 100×10^{-3} (calculated with log₁₀). The velocity constant decreases after 2 minutes and the value becomes steady again after 7 to 10 minutes, when 50 to 55 per cent of the phosphate is split off, and k is now 8×10^{-3}. Under the same conditions the hydrolysis constant k for the mixed triose monophosphates is 6×10^{-3}, nearly the same as for the second half of the phosphate of the dimeric diester (Table II).

85 mg. of the barium salt of the new ester were dissolved in about 11 cc. of water; the solution was freed from barium and neutralized. This solution contained, per cc., 1.047 mg. of organic P (1.085 mg. of total P —

0.038 mg. of inorganic P). The hydrolysis was carried out in 0.025 N hydrochloric acid at 100°. After periods of 0, 8, 15, and 180 minutes the

TABLE III

Inorganic P Formed by Acid Hydrolysis

Time of hydrolysis	P per cc.	P formed	
min.	*mg.*	*mg.*	*mM*
0	0.038		
8	0.573	0.535	17.3
15	0.630	0.592	19.0
180	1.040	1.00	32.0

Alkali-Saponifiable Organic P Formed by Acid Hydrolysis

Time of hydrolysis	Total P per cc.	Alkali-saponifiable P* formed	
min.	*mg.*	*mg.*	*mM*
8	0.925	0.352	11.3
15	1.039	0.409	13.2

Methylglyoxal Formed by Acid Hydrolysis

Time of hydrolysis	Methylglyoxal† formed	
min.	*mg. per cc.*	*mM*
0	0.00	
8	0.10	1.4
15	0.23	3.2
180	1.19	16.5
	1.21 (Maximum)	

Iodine Titration of Fischer-Baer Ester Formed by Acid Hydrolysis

Time of hydrolysis	0.01 N iodine solution per cc. hydrolysate	Glyceraldehyde 3-phosphate
min.	*cc.*	*mM*
0	0.25	
8	2.66	13.3
15	3.18	15.9
180	5.9	0.0‡

* The alkali-saponifiable organic phosphorus was determined by digestion for 15 minutes in N sodium hydroxide at room temperature (17).

† Determined colorimetrically (18).

‡ The iodine value for 180 minutes is due to methylglyoxal, while for 8 and 15 minutes only the slight excess of the calculated glyceraldehyde phosphate over the equivalence with the alkali-saponifiable P is probably attributable to the small amount of methylglyoxal (1.4 and 3.2 mM) present after that time of hydrolysis.

inorganic P, the alkali-labile organic P, the methylglyoxal, and the hypoiodite value (Willstätter and Schudel titration) were determined (Table III).

SUMMARY

The synthesis of glyceraldehyde 1,3-diphosphate (dimeric) (II) is described.

The investigation of its biological behavior by Professor O. Meyerhof and Dr. D. Drabkin has shown that a glyceraldehyde phosphate of this constitution and configuration cannot be the expected intermediary between glyceraldehyde 3-phosphate and glyceric acid 1,3-diphosphate in carbohydrate metabolism.

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0.038 mg. of inorganic P). The hydrolysis was carried out in 0.025 N hydrochloric acid at 100°. After periods of 0, 8, 15, and 180 minutes the

TABLE III

Inorganic P Formed by Acid Hydrolysis

Time of hydrolysis	P per cc.	P formed	
min.	*mg.*	*mg.*	*mμ*
0	0.038		
8	0.573	0.535	17.3
15	0.630	0.592	19.0
180	1.040	1.00	32.0

Alkali-Saponifiable Organic P Formed by Acid Hydrolysis

Time of hydrolysis	Total P per cc.	Alkali-saponifiable P* formed	
min.	*mg.*	*mg.*	*mμ*
8	0.925	0.352	11.3
15	1.039	0.409	13.2

Methylglyoxal Formed by Acid Hydrolysis

Time of hydrolysis	Methylglyoxal† formed	
min.	*mg. per cc.*	*mμ*
0	0.00	
8	0.10	1.4
15	0.23	3.2
180	1.19	16.5
	1.21 (Maximum)	

Iodine Titration of Fischer-Baer Ester Formed by Acid Hydrolysis

Time of hydrolysis	0.01 N iodine solution per cc. hydrolysate	Glyceraldehyde 3-phosphate
min.	*cc.*	*mμ*
0	0.25	
8	2.66	13.3
15	3.18	15.9
180	5.9	0.0‡

* The alkali-saponifiable organic phosphorus was determined by digestion for 15 minutes in N sodium hydroxide at room temperature (17).

† Determined colorimetrically (18).

‡ The iodine value for 180 minutes is due to methylglyoxal, while for 8 and 15 minutes only the slight excess of the calculated glyceraldehyde phosphate over the equivalence with the alkali-saponifiable P is probably attributable to the small amount of methylglyoxal (1.4 and 3.2 mμ) present after that time of hydrolysis.

inorganic P, the alkali-labile organic P, the methylglyoxal, and the hypoiodite value (Willstätter and Schudel titration) were determined (Table III).

A SYNTHESIS OF *dl*-GLYCERALDEHYDE 3-PHOSPHATE

By ERICH BAER and HERMANN O. L. FISCHER

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University of Toronto, Toronto, Canada)*

The first synthesis of *dl*-glyceraldehyde 3-phosphate (Fischer-Baer ester)
was described by the authors 11 years ago (1). A shorter synthesis has now
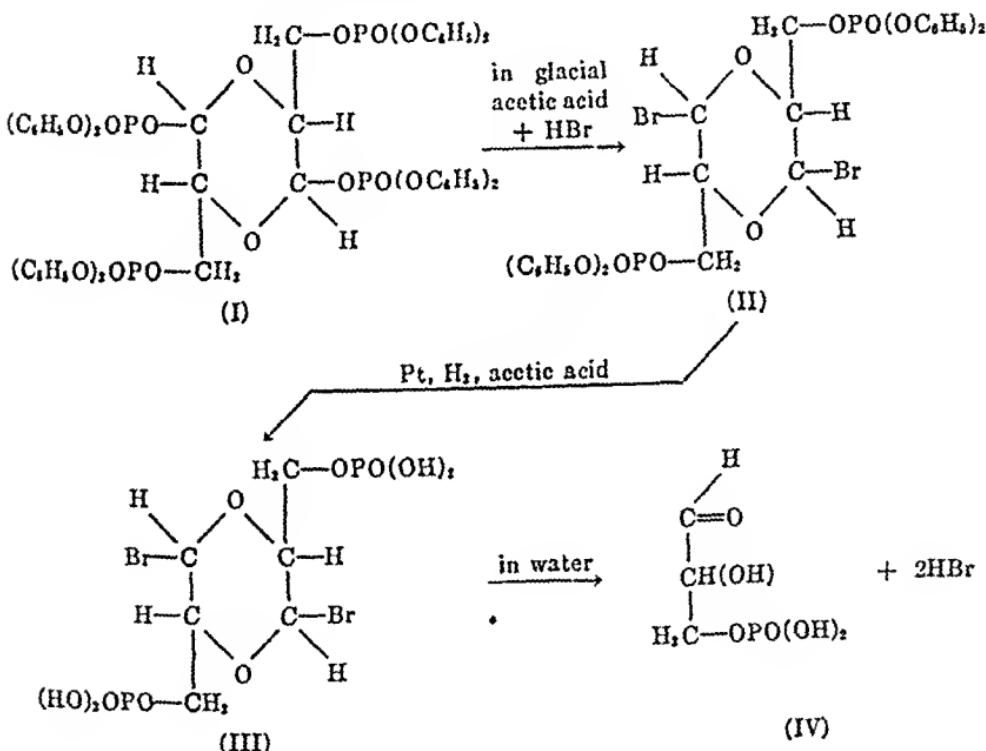
been made possible by the observation that dimeric glyceraldehyde 1,3-
bisdiphenylphosphate (I),[1] when treated with either hydrogen bromide or
hydrogen chloride in an organic solvent, exchanges its carbonyl-bound
diphenylphosphoric acid residue (in position 1) for halogen and forms in
good yield the bromide (II)[2] or the corresponding chloride. The reductive

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[1] See the preceding paper (2).

[2] This reaction is comparable with the formation of the aceto-halogen sugars from
sugar acetates.

cleavage of the glyceraldehyde 1-bromide 3-diphenylphosphate (II)[3] with platinum and hydrogen in dry acetic acid, according to the method of Brigl and Müller (3), yields dimeric glyceraldehyde 1-bromide 3-phosphoric acid (III). This substance can be purified only with great difficulty by recrystallization from the usual organic solvents. It is very easy, however, to obtain a pure dioxane addition compound of (III) by dissolving the phosphate in tributyl phosphate, and adding pure dioxane in excess. The product crystallizes in long narrow prisms, and contains 2 moles of dioxane to 1 mole of the dimeric organic phosphate (III).

When dissolved in water at room temperature, the glyceraldehyde 1-bromide 3-phosphoric acid and its dioxane compound hydrolyze rapidly, forming *dl*-glyceraldehyde 3-phosphate (IV) and hydrobromic acid. The ester was isolated in the form of its calcium salt.[4] Its identity with glyceraldehyde 3-phosphoric acid and its purity have been proved by the following determinations: C, H, P, Ca, Willstätter-Schudel titration, conversion to dinitrophenylhydrazone, formation of methylglyoxal 2,4-dinitrophenylosazone, alkali-labile phosphoric acid (4), and acid hydrolysis curve (4).

<div align="center">EXPERIMENTAL</div>

Glyceraldehyde 1-Bromide 3-Diphenylphosphate (Dimeric)—35 cc. of an ice-cold solution of hydrobromic acid[5] (30 to 32 per cent) in glacial acetic acid were added to 10 gm. of glyceraldehyde 1,3-bisdiphenylphosphate (dimeric) (I) and the stoppered flask was gently shaken until the phosphate was dissolved (3 to 5 minutes). After about 30 minutes at room temperature the first well formed crystals of the new bromo compound (II) separated. At the end of 24 hours the crystalline paste was run into 250 cc. of a well stirred mixture of ice and water (1:2) and the stirring was continued for 10 minutes. The bromo compound was filtered with suction, washed on the filter with ice-cold water, and dried *in vacuo* over phosphorus pentoxide. The crude product, weighing 6.5 gm. (93.5 per cent), when crystallized from approximately 200 cc. of boiling ethyl acetate (free from water and alcohol), yielded 5.4 gm. (77.7 per cent) of pure glyceraldehyde 1-bromide 3-diphenylphosphate (dimeric). M.p. 161–162°. The substance is readily soluble in warm benzene, toluene, carbon tetrachloride, ethyl acetate, ace-

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[3] The same reactions when carried out with the chloro compound also lead to the calcium salt of glyceraldehyde 3-phosphoric acid.

[4] If only an aqueous solution of glyceraldehyde 3-phosphoric acid is required, and the products of hydrolysis (HBr, or HCl and dioxane) do not interfere with the proposed experiment, the preparation of the calcium salt may be avoided by simply dissolving glyceraldehyde 1-bromide phosphoric acid or its dioxane compound in cold water and carefully neutralizing the solution. Since glyceraldehyde 3-phosphoric acid is rapidly destroyed in alkaline solution, it is better to stop the neutralization just before the neutral point is reached.

[5] A solution of 50 per cent hydrobromic acid in glacial acetic acid may also be used

tone, and dioxane but only sparingly soluble in warm ether or petroleum ether.

$C_{19}H_{18}O_{10}P_2Br_2$ (770.1).   Calculated.   C 46.77, H 3.67, P 8.05, Br 20.75
                    Found.   " 46.90, " 3.89, " 7.97, " 20.65

Determination of Glyceraldehyde—When 233.0 mg. of the bromo compound were refluxed for several hours with a solution of 1 gm. of 2,4-dinitrophenylhydrazine in 60 cc. of 2.5 N hydrochloric acid, 251.0 mg. of methylglyoxal 2,4-dinitrophenylosazone were obtained. This amount of osazone corresponds to 52.3 mg. of glyceraldehyde which is 96 per cent of the theoretical.

Glyceraldehyde 1-Chloride 3-Diphenylphosphate (Dimeric)—8 gm. of glyceraldehyde 1,3-bisdiphenylphosphate (I) were dissolved in 80 cc. of a 5 per cent solution of dry gaseous hydrogen chloride in pure dioxane.[6] The mixture was allowed to stand for 24 hours at room temperature. On removal of the hydrochloric acid and dioxane *in vacuo* (bath 25°) a crystalline substance was obtained. The residue was triturated with 25 cc. of ice-cold methanol, filtered with suction, washed on the filter twice with 8 cc. of cold methanol, and immediately dried *in vacuo*. Yield 3.9 gm. (79.3 per cent). The chloro compound was dissolved in 50 cc. of lukewarm chloroform (free from ethanol) and precipitated by adding 70 cc. of petroleum ether (b.p. 60–80°). This process was repeated, if necessary, until a melting point of 146–147° was reached.

$C_{10}H_{21}O_{10}P_2Cl_2$ (681.5).   Calculated.   C 52.9, H 4.14, Cl 10.42, P 9.11
                    Found.   " 52.7, " 4.24, " 10.35, " 9.33

The chloro compound when treated with an excess of 2,4-dinitrophenylhydrazine in boiling 2.5 N hydrochloric acid yielded 97 per cent of methylglyoxal 2,4-dinitrophenylosazone.

Glyceraldehyde 1-Bromide 3-Phosphoric Acid (Dimeric)—A suspension of 2.3 gm. of finely powdered glyceraldehyde 1-bromide 3-diphenylphosphate (m.p. 161–162°) and 0.60 gm. of platinum oxide[7] in 50 cc. of dry acetic acid[8] was vigorously shaken in an atmosphere of pure, dry hydrogen at a pressure of approximately 60 cm. of water. During the entire cleavage the reaction mixture was kept at about 15–20°.[9] The reductive cleavage was finished

[6] A 30 per cent solution of gaseous hydrogen chloride in dry acetic acid may be used according to the procedure given for the bromo compound.

[7] The time for the reductive cleavage of the phenyl ester may be considerably shortened by the use of a larger amount of catalyst. It seems, however, that with increasing amounts of catalyst the formation of apparently non-cleavable cyclohexyl esters is favored. The presence of such cyclohexyl esters which are difficult to remove later on is indicated by too high carbon values.

[8] The glacial acetic acid of high quality was boiled under a reflux with chromic acid anhydride, distilled, and kept over drierite. Traces of the drying reagent were removed immediately before use by centrifuging.

[9] The reaction mixture was cooled by dropping ether on the reaction vessel.

in approximately 4 to 5 hours, at the end of which time 1161 cc. of hydrogen[10] (17.4 moles) had been absorbed. After the hydrogen was replaced with nitrogen, the platinum catalyst was filtered off and washed several times with a few cc. of dry acetic acid. The combined filtrates were brought to dryness *in vacuo* (10 mm.) at a bath temperature not higher than 25°. The crystallized residue was mixed with 5 cc. of dry acetic acid, filtered with suction, and washed on the filter three times with 3 cc. of acetic acid. The substance, dried *in vacuo* over phosphorus pentoxide and solid sodium hydroxide, weighed 1.1 gm. (79.1 per cent). At this stage according to the determination of C, H, total P, alkali-labile P, and HCl-labile P the substance was 90 to 92 per cent pure. Because the substance has unfavorable solubilities and is very readily hydrolyzed, it was difficult to purify it by recrystallization.

A somewhat purer compound was obtained as follows: 1.0 gm. of the bromo compound was boiled under a reflux in 40 cc. of a 4 per cent solution of dry acetic acid in dry acetone for a period of 30 minutes. The material was filtered with suction and washed on the filter with dry acetone. A small amount of material of a slightly pink color was removed on the filter by floating it off with acetone. The rest of the substance was dried in a high vacuum (recovery 70 per cent). The substance is very slightly soluble in boiling dry acetone, benzene, ethyl acetate, tetrachloroethane, but easily soluble in trimethyl phosphate and tributyl phosphate.

$C_6H_{12}O_{10}P_2Br_2$ (466)

     Calculated.   C 15.46, H 2.60, P 13.30, Br 34.3
     Found.   " 15.7, 15.85, H 2.98, 2.80, P 13.03, 13.0, 13.30, Br 33.8

The substance contained traces of inorganic phosphate which were too small to be determined. No appreciable amount of inorganic phosphate was formed after the dry substance had been kept in an ice box for more than a month. The sensitivity of the compound towards acid or alkali is equal to that of its product of hydrolysis; namely, glyceraldehyde 3-phosphoric acid. Thus the action of 1 N hydrochloric acid in 1 hour at 100° or 1 N sodium hydroxide in 20 minutes at room temperature liberated 98 and 94 per cent respectively of the phosphoric acid.

Glyceraldehyde 1-bromide 3-phosphoric acid may be best obtained pure in the form of its dioxane addition compound.

Dioxane Compound of Glyceraldehyde 1-Bromide 3-Phosphoric Acid (*Dimeric*)—To a solution of 1 gm. of glyceraldehyde 1-bromide 3-phosphoric acid in 5.5 cc. of tributyl phosphate[11] were added 11 cc. of pure di-

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[10] Corrected to standard conditions. Allowances were made for the hydrogen necessary for the reduction of the catalyst.

[11] Obtained from the Commercial Solvents Corporation.

oxane. The mixture was kept at room temperature for a period of 20 hours, during which time the dioxane compound crystallized spontaneously in long narrow prisms. It seems that a purer substance is obtained by allowing the crystallization to proceed slowly and undisturbed. The mother liquor was carefully decanted and the substance was washed with dioxane. After being dried in a high vacuum at room temperature over solid sodium hydroxide 1.2 gm. (87 per cent) of the dioxane compound were obtained. The material was *completely free* from inorganic phosphate. If kept cool and dry the substance is stable and can be stored without decomposition over a considerable period of time; *e.g.*, after standing in an ice box for 2 months not more than 1.8 per cent of the total organic phosphate had become inorganic phosphate.[12]

$C_3H_{12}O_6P_2Br_2 + 2C_4H_8O_2$ (642)
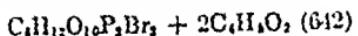
Calculated.	C 26.2,*	H 4.39,	P 9.65,	Br. 24.90	
Found.	" 26.0,*	" 4.33,	" 9.76, 9.64,	Br 24.80, 24.95	

* Correct carbon values could be obtained only by weighing the substance in a closed vessel and starting the combustion immediately after the substance is mixed with dichromate (otherwise loss of dioxane would result); *cf.* behavior of addition compounds of dioxane with oxalyl chloride (5).

The action of 1 N hydrochloric acid at 100° in 1 hour or 1 N sodium hydroxide at room temperature in 20 minutes liberates 99.4 and 96.0 per cent respectively of the phosphoric acid.

When the dioxane compound was refluxed with an excess of 2,4-dinitrophenylhydrazine in 2.5 N hydrochloric acid for 1 hour, 98.3 and 100.1 per cent of methylglyoxal 2,4-dinitrophenylosazone were obtained.

The glyceraldehyde 1-bromide 3-phosphoric acid and its dioxane compound hydrolyze readily, forming glyceraldehyde 3-phosphate which can be isolated in the form of its calcium salt.

Calcium Glyceraldehyde 3-Phosphate—An ice-cold solution of 1.0 gm. of the glyceraldehyde 1-bromide 3-phosphate dioxane compound in 3.3 cc. of water was mixed with a cold solution of 1.1 gm. of calcium acetate in 5.5 cc. of water and immediately filtered. To the filtrate, which was kept in ice, were added 6.3 cc. of 95 per cent ethanol drop by drop with scratching in the course of 30 minutes.[13] After the mixture was allowed to stand in ice for a short time, the calcium salt was filtered with suction. It was thoroughly freed from mother liquor, washed three times with 3 cc. of ice-cold 40 per cent ethanol, and dried as much as possible on the filter by suction. A pure dihydrate of the calcium salt was obtained by drying the pulverized

[12] Since the substance is stable, it can be stored and, if need arises, quickly converted into the calcium salt of glyceraldehyde 3-phosphoric acid.
[13] In order to obtain a better crystallized calcium salt the alcohol has to be added slowly, especially at the beginning.

substance at room temperature *in vacuo* (8 to 10 mm.) over calcium chloride for 2 hours.   Yield 0.48 gm. (63 per cent).   The calcium salt contained only traces of inorganic phosphate.

When the following analyses were carried out, the best results were obtained when the substance was analyzed immediately after its preparation.
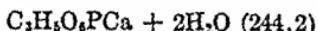
$C_3H_5O_6PCa + 2H_2O$ (244.2)
         Calculated.   C 14.75, H 3.71, P 12.7, Ca 16.4
         Found.   " 14.7, 14.6, H 4.2, 4.3; P 12.7, 12.6, Ca 16.2

Titration of Aldehyde According to Willstätter and Schudel (6)—A solution of 111.7 mg. of the calcium salt of glyceraldehyde 3-phosphoric acid in a mix-
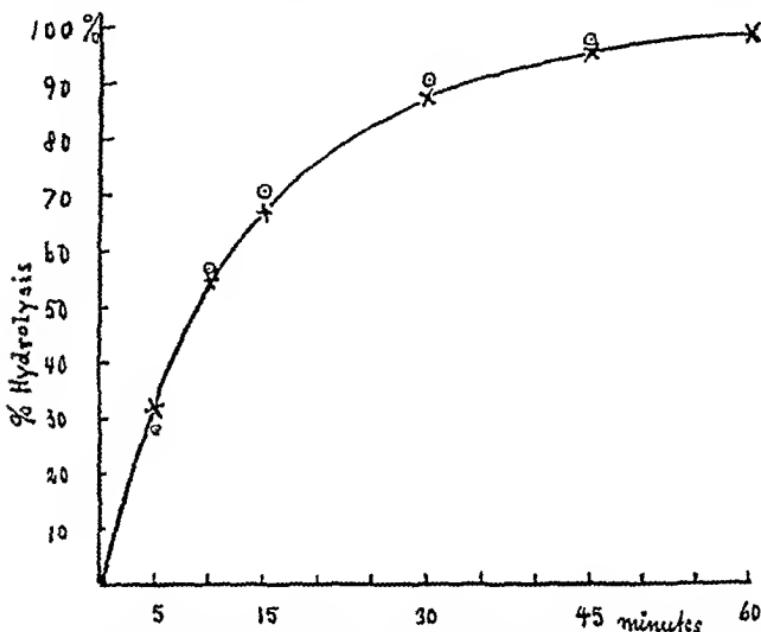
Fig. 1. Acid hydrolysis of an approximately 0.002 M solution of glyceraldehyde 3-phosphate.   The values represented by circles were determined by Kiessling (7).

ture of 100 cc. of water, 30 cc. of 0.10 N hydrochloric acid, and 15.00 cc. of 0.10 N iodine solution was made alkaline by adding drop by drop 50 cc. of 0.10 N sodium hydroxide solution.   After 30 minutes it was acidified and the iodine titrated.   9.10 cc. (99.6 per cent of the theoretical) of 0.10 N iodine solution had been used up.

Glyceraldehyde 3-Phosphoric Acid 2,4-Dinitrophenylhydrazone—To an ice-cold solution of 108 mg. of calcium salt in a few drops of 2 N hydrochloric acid was added a solution of 80 mg. of 2,4-dinitrophenylhydrazine in 5 cc. of 2 N hydrochloric acid.[14]   The hydrazone, which precipitated immediately,

14 The hydrazine solution should be just warm enough to keep the dinitrophenylhydrazine hydrochloride in solution.

was centrifuged off, washed on the centrifuge once with a small volume of 2 N hydrochloric acid, and dried at room temperature in a high vacuum over phosphorus pentoxide and sodium hydroxide.[15]

$$C_9H_{11}O_9PN_4 \text{ (350.2).} \quad \text{Calculated, N 16.0; found, N 15.96}$$

Methylglyoxal 2,4-Dinitrophenylosazone—Refluxing 97.1 mg. of glyceraldehyde 3-phosphate with an excess of 2,4-dinitrophenylhydrazine in 2 N hydrochloric acid for 1 hour yielded 172.7 mg. (100.5 per cent) of methylglyoxal 2,4-dinitrophenylosazone.

Alkali-Labile Phosphate (4)—110.6 mg. of calcium salt were dissolved in 3 cc. of 0.10 N hydrochloric acid and the solution was made up to a volume of 100.0 cc. with 1 N sodium hydroxide. After the solution had stood at room temperature for 20 minutes, the inorganic phosphoric acid was determined colorimetrically and found to be 41.50 mg. or 93.0 per cent of the organic phosphate.

Acid Hydrolysis (4)—An approximately 0.002 M solution of glyceraldehyde 3-phosphate in 1 N hydrochloric acid was hydrolyzed at 100° and the inorganic phosphate which was formed after 5, 10, 15, 30, 45, and 60 minutes was determined. The hydrolysis is given in Fig. 1.

SUMMARY

A second and shorter synthesis of glyceraldehyde 3-phosphate is described.

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[15] The isolation of the hydrazone must be carried out as rapidly as possible to avoid the formation of appreciable amounts of methylglyoxal 2,4-dinitrophenylosazone.

THE GASOMETRIC DETERMINATION OF FREE AMINO ACIDS IN BLOOD FILTRATES BY THE NINHYDRIN-CARBON DIOXIDE METHOD

By PAUL B. HAMILTON AND DONALD D. VAN SLYKE

WITH THE TECHNICAL ASSISTANCE OF MISS SONIA LEMISH

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

For determination of free amino acids in biological material measurement of the CO_2 formed by their reaction with ninhydrin has been found to be the most specific method available (1, 2). It is in fact so specific that MacFadyen (3) was able to ascertain conditions under which it can be used to determine the amino acid content of plasma without removal of the proteins, and this procedure was successfully applied in clinical and physiological investigations by MacFadyen and others, and in routine clinical analyses in this laboratory.

We find, however, that under properly chosen conditions the proteins can be removed without loss of amino acids, and that their removal prior to the ninhydrin-CO_2 analysis makes it possible to eliminate certain corrections and steps in manipulation, and thereby to increase both the speed and the accuracy of the determinations. Analysis of protein-free filtrates is now the procedure used in this laboratory, both for clinical routine and for experimental work.

As protein precipitants colloidal iron, zinc hydroxide, cadmium hydroxide, tungstic acid, sulfosalicylic acid, p-toluenesulfonic acid, and picric acid have been tried. Picric and tungstic acids both gave quantitative yields of α-amino nitrogen,[1] but picric acid proved the more convenient. Its application is simple, and it yields a filtrate of pH 1.8 to 2.0, which is suitable without addition of other buffer for the ninhydrin reaction. Tannic acid and trichloroacetic acid cannot be used because when present in the filtrates they decompose with evolution of CO_2 during the course of the reaction with ninhydrin at 100°. The heavy metal precipitants used at room temperature gave low results and incomplete recoveries.

The ninhydrin which serves to set free the CO_2 from the amino acid

[1] The term "α-amino nitrogen" is used to indicate values calculated as 1 atom of nitrogen per molecule of CO_2 evolved by reaction with ninhydrin (2). In hydrolysates of animal proteins the α-amino nitrogen thus estimated is about 70 per cent of the total N, the rest being the amide N and the non-α-N of the hexone bases and tryptophane. Probably the total amino acid N of blood plasma is about 1.5 times the α-amino nitrogen, because of the presence of amino acids with non-α-nitrogen.

carboxyl groups serves also to remove urea from the sphere of action. Urea is hydrolyzed by water at 100° and pH 1 to 5 at such a rate that CO_2 equivalent in terms of α-amino nitrogen to 1 per cent of the urea nitrogen is set free by the hydrolysis every 10 minutes. Consequently in the 20 minutes used for the ninhydrin reaction in blood filtrates hydrolysis of urea would yield CO_2 indicating α-amino nitrogen equal to 2 per cent of the urea nitrogen, if no reagent were added to retard the hydrolysis. Fortunately ninhydrin itself acts as such a reagent. As shown by the writers (4), ninhydrin combines with urea in water solution to form a compound which resists hydrolysis by hot water at the acidities used. When, as in routine analyses of non-uremic blood, the picric acid filtrates are at once heated for 20 minutes after addition of 20 mg. of ninhydrin per cc., combination of ninhydrin and urea takes place so rapidly that the CO_2 formed in the 20 minutes from urea corresponds to α-amino nitrogen equal to only 0.7 per cent of the urea nitrogen. This proportion is so small that the CO_2 from urea can be treated as a constant correction, equivalent to 0.1 mg. of amino acid nitrogen per 100 cc. of plasma, when the blood urea nitrogen is within the ordinary normal limits of 5 to 20 mg. per 100 cc. When retention raises the blood urea to pathological levels, it is necessary only to double the amount of ninhydrin used in the analysis and incubate the blood filtrate and ninhydrin at 60° for 3 hours to complete the combination between urea and ninhydrin. Subsequent heating for 10 minutes at 100° gives a quantitative yield of CO_2 from the amino acid carboxyl groups, while the urea evolves no significant amounts during either the preliminary period at 60° or the final heating at 100°.

<center>APPARATUS</center>

The *Van Slyke-Neill manometric apparatus, storage vessel for CO_2-free 0.5 N NaOH, alundum pieces* to promote smooth boiling, *calibrated glass spoons* for measuring 100 mg. of ninhydrin, *water bath* for reaction vessels, and *test-tube rack or wire basket* for holding the reaction vessels are all as described for the manometric ninhydrin-CO_2 method by Van Slyke, Dillon, MacFadyen, and Hamilton (2), or for the manometric carbon determination by Van Slyke and Folch (5). *The automatic attachment for raising and lowering the mercury* in the manometric chamber, described on pp. 645 and 524 of these papers (2, 5), is particularly convenient if numerous series of analyses are to be carried out. To the *reaction vessel* (2) improvements, described below, have been made for facilitating precision.

All-Glass Reaction Vessel—The vessel shown in Fig. 1, A[2] has only two parts and one ground joint, is compact, and in manipulation has proved

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[2] Reaction vessels, as shown in Fig. 1, A, of Pyrex glass are made by E. Machlett and Son, 220 East 23rd Street, New York.

more convenient than the all-glass vessel previously described ((2) Fig. 1, C). For maximal accuracy, in microdeterminations of the 0.03 to 0.05 mg. of α-amino nitrogen usually measured in plasma analyses, it is desirable to avoid contact of rubber with the gases in the reaction vessel. In the vessel shown in Fig. 1, A we have without error let the gases produced by the reaction stand for several days before determining the CO_2 evolved. For the most precise general work with the manometric ninhydrin-CO_2 method we recommend this vessel in place of those exposing even slight rubber surfaces.

Essential for use of the vessel in Fig. 1, A is a lubricant for the stopper which will maintain an efficient seal during the heating in the bath, and yet will not be too stiff when cooled to room temperature. A cheap lubricant[3] has been found which has almost the same viscosity at 100° as at room temperature, and joints sealed with it will stand hours of immersion in boiling water without leaking. Glycerol becomes so fluid at 100° that a seal made with it may fail to last through the 20 minute heating used in the blood filtrate analysis.

The errors that may arise from passage of CO_2 to and from the rubber pores of reaction vessels with inner rubber surfaces have been discussed by Van Slyke et al. ((2) foot-notes on pp. 632 and 635), and reaction vessels have been designed by these authors (2) and by MacFadyen (3) which retain the rubber joints with their convenience in mobility and freedom from lubricants, and yet expose only minimal rubber surfaces to the gases in the vessel. These vessels are satisfactory for "macro" and "micro" determinations of 0.2 mg. or more of α-amino nitrogen (2); but when used for "submicro" (2) determinations of 0.05 mg. or less of α-amino nitrogen, as in blood analyses, special precautions are required to keep the "rubber blank" small and so constant that it does not cause significant errors (3). Also, to avoid measurable errors from CO_2 diffusion to and from rubber in such analyses, the CO_2 must be determined in each analysis immediately after its evolution; hence one could not carry out the ninhydrin reaction in a number of vessels together and determine the CO_2 in the series at leisure later. The all-glass reaction vessel shown in Fig. 1, C of Van Slyke, Dillon, MacFadyen, and Hamilton (2) overcomes these disadvantages but involves the expense and inconvenience of two ground glass joints.

Alternative Reaction Vessel Closed with Rubber-Jointed Adapter Protected by Mercury—Fig. 1, *B* shows an alternative vessel which can be readily made in any laboratory with glass-blowing equipment. The vessel is preferably of Pyrex glass. The adapter consists of a rubber tube, of 12 mm. outer diameter and 5 mm. bore, which slips over the neck of the vessel and is closed by a glass plug applied as described for a somewhat similar adapter by MacFadyen ((3) p. 389 under "Technique of handling reaction vessel in analyses"). Enough mercury is placed in the vessel so that when it is inverted the rubber joint is covered with a layer of mercury at least 2 cm. deep. By keeping the vessel inverted during the reaction, and after it, until the CO_2 evolved is determined, loss or gain of CO_2 by the rubber is prevented. This vessel with care is usually as accurate as the all-glass vessel but more liable to occasional small error.

Rubber Connecting Tubes—To connect either vessel with the Van Slyke-Neill chamber during transfer of CO_2 a 5 cm. length of stethoscope tubing is used, of $\frac{1}{2}$ inch (12 mm.) outer diameter and $\frac{1}{8}$ inch (3 mm.) bore. It is necessary to remove the CO_2 from the pores of the rubber before use. The tube is cleaned inside with a test-tube brush and is immersed in acidified water in a round bottomed flask and boiled for 30 minutes. The flame is then removed, and the flask is immediately stoppered and cooled under the tap. The vacuum formed in the flask by condensation of steam draws the residual gases from the pores of the rubber. After the bubbles stop coming out of the rubber, the flask is opened and the tubes are washed with distilled water. As many tubes are prepared together as are needed for the vessels used. If the rubber tubes are used with the all-glass reaction vessel (Fig. 1, *A*), repetition of the treatment is unnecessary.

If the adapter tube of Fig. 1, *B* is used daily, repetition of the treatment is also unnecessary, but if it stands more than a day or two in the air CO_2 may reaccumulate in the pores and must be removed. This reaccumulation of CO_2 varies with the kind and quality of rubber tubing employed. The rates at which CO_2 is evolved at 100° *in vacuo* and reaccumulated at room temperature in air can easily be determined. A 5 cm. length of air-exposed, untreated tubing is placed in an all-glass reaction vessel (Fig. 1, *A*) with 5 cc. of 0.01 N HCl. The reaction vessel is evacuated, heated for 10 minutes in the 100° bath, and the CO_2 evolved from the piece of rubber is determined as described in the sections on absorption of CO_2 and determination of CO_2. This process of 10 minute heating followed by removal and measurement of the evolved CO_2 is repeated until the CO_2 evolution becomes nil or reaches a low and constant value.' The rubber tube is then left exposed for several days and again analyzed. If CO_2 reaccumulates or is picked up from the atmosphere, an increase over the low constant value previously determined will indicate the amount of CO_2 reaccumulated during the period of exposure. The results obtained indicate the comparative behavior of different tubings, but do not show the much smaller magnitude of error caused by exposure of the small surface in contact with the gases when an adapter is used (*e.g.*, as in Fig. 1, *A* or *B* of Van Slyke *et al.* (2)). In analyses the error produced by a rubber adapter is usually negative. The rubber is likely to take

up CO_2 evolved by the amino acids, lowering the measured yield. But when blank analyses are carried out, the rubber gives off CO_2, and causes a plus error in the c correction determined in the blank. Both errors are additive in causing low results for α-amino nitrogen.

Reagents—

Ninhydrin,[4] pulverized.

Approximately 0.5 N NaOH of minimal CO_2 content in nearly saturated NaCl solution. This is prepared as described on p. 637 of Van Slyke, Dillon, MacFadyen, and Hamilton (2), except that, for dilution of the concentrated NaOH to 0.5 N strength, CO_2-free 25 per cent NaCl solution (250 gm. of NaCl to 750 cc. of water) is used in place of water.[5]
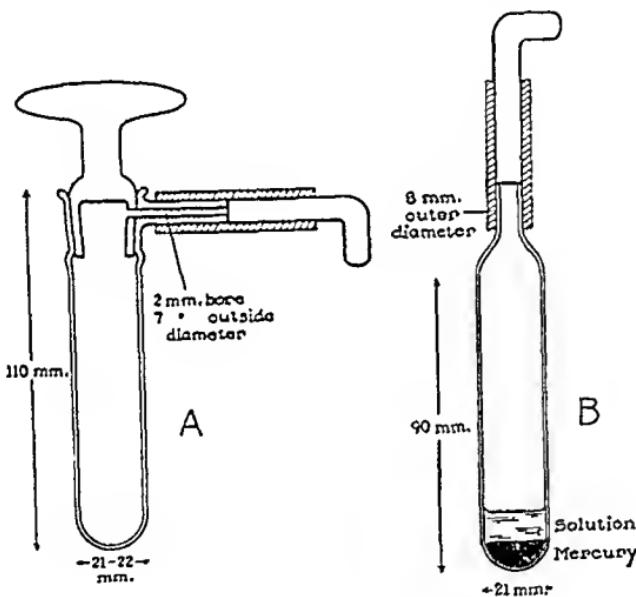
Fig. 1. Reaction vessels

Approximately 5 N NaOH. Prepared by mixing 1 volume of the 18 to 20 N NaOH with 3 volumes of water.

Approximately 2 N lactic acid in nearly saturated NaCl solution. 1 volume of concentrated lactic acid (sp. gr. 1.20) is diluted to 5 volumes with the 25 per cent NaCl solution.[5]

[4] The ninhydrin at present used is purchased from the University of Illinois, Urbana, Illinois.

[5] As found by MacFadyen (3), in microdeterminations of CO_2 the use of approximately saturated salt solutions in the manometric chamber increases accuracy because it retards reabsorption of CO_2 during the compression of the gas volume from 47 cc. to 0.5 cc. in the analysis, so that instead of a correction for reabsorption of 3.7 $\pm$ 0.5 per cent of the CO_2 a correction of only 0.6 $\pm$ 0.1 per cent is needed.

1 per cent (0.0437 N) picric acid solution. About 15 gm. of 90 per cent picric acid (10 per cent moisture), or 12 gm. dry, are dissolved with warming in 1 liter of water. 20 cc. are titrated with 0.1 N NaOH to the first color change of phenolphthalein. The titration figure will be about 10 cc. of the NaOH. The volume of water that is needed to dilute the picric acid solution so that the titration figure will be 8.73 cc. is calculated and added.

PROCEDURE FOR BLOOD OF NORMAL UREA CONTENT

Handling of Blood Sample—2 cc. of plasma or 1 cc. of whole blood or cells provides sufficient material for duplicate analyses with an error less than 1 per cent. For a single analysis of plasma with an error not over 2 per cent a sample of 0.5 cc. may be used.

When plasma is analyzed, the blood is centrifuged soon after being drawn, and hemolysis is avoided. The α-amino nitrogen in cells is 1.5 to 2 times as concentrated as in plasma, and plus errors in analysis of plasma may result if hemolysis or injury of cells permits their amino acids to diffuse into the plasma.

Plasma rather than serum must be analyzed if one wishes to determine the amino acid content of the circulating plasma. As shown by Mac-Fadyen (3), when blood coagulates amino acids are set free by reactions not yet understood, so that the α-amino nitrogen of serum may be 20 per cent higher than the α-amino nitrogen of uncoagulated plasma. Hence anticoagulant, 0.1 or 0.2 mg. of heparin or 1 mg. of oxalate per cc. of blood, is added as soon as the blood is drawn.

Precipitation of Proteins of Plasma—1 volume of plasma is pipetted into a round bottomed centrifuge tube and 5 volumes of 1 per cent picric acid solution are added. The mixture is vigorously shaken. Complete precipitation of the proteins is effected in a few seconds.

When sufficient plasma is available, 2 cc. are precipitated with 10 cc. of the picric acid, and sufficient supernatant is obtained for duplicate analyses on 5 cc. aliquots. However, a single analysis with an error usually less than 2 per cent can be performed with 0.5 cc. of plasma. In this case the precipitation is made with 5.5 cc. of picric acid and for calculation the factors in Table I are doubled.

Precipitation of Proteins of Whole Blood or Cells—The sample of *whole blood* is diluted in a round bottomed centrifuge tube with *an equal volume* of water. The sample of *cells* is diluted with *2 volumes* of water. 1 volume of the diluted laked solution of either whole blood or cells is mixed with 5 volumes of picric acid, as described for plasma. The mixture is shaken vigorously until the color changes from reddish to yellow, indicating complete precipitation. Shaking for about 1 minute suffices.

Centrifugation of Protein Precipitate—The mixture is centrifuged for 10

minutes at 3000 R.P.M., and the supernatant solution is decanted through a funnel containing a plug of cotton about the size of a pea (a larger plug, or a paper filter, might absorb too much filtrate to leave enough for duplicate analyses). Portions of 5 cc. each are taken for analysis.

Removal of Preformed and Labile CO_2—The filtrates contain some dissolved CO_2 from the blood bicarbonate and occasionally substances that break down to form CO_2 on heating to 100° ("labile CO_2"). To remove CO_2 from these sources the 5 cc. sample of filtrate is pipetted into a reaction vessel, two or more alundum pieces and a drop of octyl alcohol are added, the filtrate in the open vessel without stopper or adapter is heated to boiling in about 0.5 minute over the free flame of a micro burner, and is then boiled[6] for exactly 1 minute. If boiling is continued longer than 1 minute, the α-amino nitrogen may be measurably reduced.[7] After the boiling the vessel is set into cold water until the filtrate is cooled below 25° (3 minutes in cold tap water or 1.5 minutes in ice water).

Addition of Ninhydrin and Evacuation of Air—If the *all-glass reaction vessel* is used, one of the short rubber connecting tubes (Fig. 1, *A*) is lubricated by dipping in water and slipped on the side arm, the ground glass surface is wiped dry, 100 mg. of ninhydrin[8] measured from a calibrated glass spoon are added through a short-stem funnel, and the stopper, its ground surface previously covered with a thin film of lubricant, is set into place with the opening through the side tube open. The vessel, as quickly as possible after the ninhydrin is added (see pp. 640, 641 (2)), is evacuated to 30 mm. or less pressure. Tilting and shaking gently during the first few seconds of evacuation prevent loss of solution from undue effervescence; if necessary the speed of evacuation may be controlled by partial closure of the ground glass stopper. After evacuation is completed, the vessel is closed by rotating the stopper through 180°. The rubber tube (Fig. 1, *A*) is pressed flat between thumb and forefinger as the connection with the

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[6] The "100° CO_2," to remove which from whole plasma MacFadyen (3) used a 10 minute preheating period, is entirely removed from the filtrate by the 1.5 minute heating.

[7] Hamilton (7) has found that 20 per cent or more of the α-amino nitrogen of normal plasma is in the form of a substance which behaves like glutamine. Glutamine undergoes ring formation to yield pyrrolidonecarboxylic acid

$$NH_2 \cdot CO \cdot CH_2 \cdot CH_2 \cdot CH(NH_2) \cdot COOH \rightarrow \underbrace{CO \cdot CH_2 \cdot CH_2 \cdot CH(NH)} \cdot COOH + NH_3$$

on heating at pH 2. The pyrrolidonecarboxylic acid, where the $CH(NH_2) \cdot COOH$ group is replaced by a $CH(NH-COR) \cdot COOH$, as in acetylated amino acids, gives off no CO_2 when heated with ninhydrin. The decrease in α-amino N of the plasma filtrate that occurs from the ring formation during 1.5 minutes heating is not significant, but longer heating would make it significant.

[8] In analysis of blood of high urea content 200 mg. of ninhydrin instead of 100 mg. are added (see the section "Procedure for blood of high urea content").

pump is broken, in order to keep air out of the side tube, and the glass plug, lubricated with distilled water or glycerol, is inserted as shown in Fig. 1, *A*, trapping as little air as possible. (If the plug were pushed in against the pressure of air, expansion of the air might later force the plug out in the boiling bath.) It is a desirable precaution to secure the ground glass stopper by anchoring it to the side arm with several turns of a stout elastic band. This will prevent the glass stopper from being forced out during the boiling, as it might otherwise be if macro amounts of CO_2 are determined or if the reaction vessel has not been evacuated sufficiently.

If the vessel with adapter (Fig. 1, *B*) is used, 2 cc. of mercury are first added, then the ninhydrin. The rubber adapter tube is lubricated by dipping the end in water and is slipped over the neck of the reaction vessel. The vessel is then evacuated and is closed temporarily by a pinch clamp on the rubber tube, and the pump is disconnected. The rubber tube is then pinched flat, between thumb and finger, and the glass plug is inserted, as described in the above paragraph. The clamp is then removed and the glass plug pushed down into contact with the neck of the vessel, which is then as shown in Fig. 1, *B*. (The technique, introduced by MacFadyen (3), is more completely described on p. 389 of his paper.)

Immersion in Water Bath at 100°[9]—The vessels, if of the all-glass type, are placed upright in a cylindrical test-tube rack or a wire basket. If the vessel with adapter (Fig. 1, *B*) is used, however, it is *inverted*, so that the mercury covers the rubber joint and forms a layer about 2 cm. deep over it. The vessels are *completely* immersed in an *actively* boiling bath for 20 minutes. Exactly at the end of this time they are removed from the bath. Glass stoppers are given a turn of 5° or 10° to insure tight sealing. The vessels are cooled to room temperature and their CO_2 contents are determined when convenient. If the adapter-fitted vessels are used, they are kept inverted until the CO_2 is determined, in order to prevent any diffusion of CO_2 into the rubber of the joint.

Absorption of CO_2 by Alkali in Van Slyke-Neill Chamber—This is accomplished as described on pp. 643-646 of Van Slyke, Dillon, MacFadyen, and Hamilton (2), but with modification in the following details. The chamber is charged with 2 cc. of the 0.5 N NaOH nearly saturated with NaCl instead of the 0.5 N NaOH employed by Van Slyke, Dillon, *et al.* The glass plug is then removed from the rubber tube on the side arm of the reaction vessel, and the side arm is reevacuated at the pump for a few

[9] In analysis of blood of high urea content the heating at 100° is preceded by incubation for 3 hours at 60°, or overnight at 37°, to combine urea and ninhydrin (see the section "Procedure for blood of high urea content"). Also, the heating period at 100° is shortened to 10 minutes.

seconds, in order to remove any CO_2 that may have evolved from the rubber and collected in the side arm during the boiling. The tube is then pinched flat between the fingers and slipped over the side arm of the Van Slyke-Neill chamber. The transfer of CO_2, as noted by MacFadyen (3), requires only half as many excursions of the gases to and from the manometric chamber as when 0.5 N NaOH without NaCl is used. Hence five excursions suffice if the filtrate is warmed to 38°, or ten excursions if it is at room temperature (20–25°). Temperature above 38° is avoided, or enough water will distil over into the alkali to increase appreciably its volume, and introduce a small error in calculations based on an S volume of 3 cc. The first excursion of the mercury is made carefully to prevent any of the reaction mixture from bumping over into the chamber of the Van Slyke-Neill apparatus.

Determination of CO_2 in Manometric Apparatus—The technique is as described previously ((2) p. 646), except that the 2 N lactic acid added is nearly saturated with NaCl, and that to extract the CO_2 after addition of the lactic acid it is necessary to shake the solution *3 minutes* instead of the 1.5 minutes which suffice when reagents without NaCl are used in the chamber. The salt accelerates the absorption of CO_2 by the alkali, as mentioned above, but it retards evolution of the CO_2 after acidification.

Blank Analysis for c Correction—It is not necessary to add the ninhydrin or to heat in the water bath. One merely places 5 cc. of the 1 per cent picric acid solution in a reaction vessel, and boils it for 1 minute to remove dissolved atmospheric CO_2. The solution is cooled and evacuated, and the analysis is carried through from the point of "Absorption of CO_2 by alkali in Van Slyke-Neill chamber." The c correction is the value of $p_1 - p_2$ found in the blank analysis. All but 1.5 ± 0.5 mm. of the CO_2 pressure measured at 0.5 cc. volume in the blank analysis is due to traces of carbonate in the 0.5 N NaOH. This carbonate is usually enough to cause a pressure of 20 to 30 mm. at 0.5 cc. volume. For a given NaOH solution it is constant, provided the solution is guarded from atmospheric CO_2 by a soda lime tube. The c correction should not be over 30 mm. if the 0.5 N NaOH is prepared as directed.

PROCEDURE FOR BLOOD OF HIGH UREA CONTENT

If urea nitrogen exceeds 20 mg. per 100 cc. of blood or plasma, the CO_2 evolved by hydrolysis of the urea in the routine α-amino nitrogen analysis described above will be equivalent to more than $20 \times 0.007 = 0.14$ mg. of α-amino nitrogen per 100 cc., and the routine procedure will require modification for exact results. One may choose the most convenient of three procedures: (A) Simply by doubling the amount of ninhydrin added and incubating at 37° or 60° before heating at 100° the urea is combined

with ninhydrin and prevented from evolving significant amounts of CO_2. (B) The urea is removed with urease. (C) The urea is determined in a separate analysis and 0.007 of its nitrogen is subtracted from the α-amino nitrogen determined by the routine procedure.

A. Combination of Urea with Excess Ninhydrin—The routine procedure is followed unchanged except at the following points. Instead of 100 mg. of ninhydrin 200 mg. are used. The evacuated reaction vessel is warmed to 60° and shaken gently for a minute to dissolve the ninhydrin and then incubated 3 hours at 60°, or overnight (16 hours) at 37°, in order to obtain maximal combination of the urea and ninhydrin (4). (If the reaction vessel with adapter and mercury seal is used (Fig. 1, B), it is inverted during the incubation.) After this treatment the vessel is heated 10 minutes in the boiling water bath. The 10 minute boiling suffices with the high ninhydrin concentration, but no harm is done if the routine 20 minute heating is used.

Except for the use of more ninhydrin and the incubation, the analysis is the same as the routine procedure with blood of normal urea content, and the c correction determined in blank analysis is the same. This procedure, *which requires no additional reagents, and no extra manipulation except the incubation,* will ordinarily be found the most convenient for handling bloods with high urea content.

B. Removal of Urea with Urease—2 mg. of Squibb's Double Strength urease[10] per cc. are stirred into the plasma or diluted blood, and the urease is permitted to act for an hour at 37° or 2 hours at room temperature above 20°.[11]

For precipitating reagent 10 gm. of citric acid, $C_6H_8O_7 \cdot H_2O$, per liter are added to the 1 per cent picric acid solution; otherwise in blood of very high urea content the amount of ammonia formed would neutralize the picric acid and prevent complete precipitation of the proteins.

When urease is used, blank analyses must be carried out in which urease is added to water instead of blood, and the analysis, including heating with ninhydrin, is carried through, as with blood. As shown by Archibald and

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[10] The urease should be standardized as described by Van Slyke and Cullen (8, 9) or in the accompanying paper by Van Slyke, MacFadyen, and Hamilton (10). If 1 mg. of the urease hydrolyzes urea at a rate slower than 0.1 mg. per minute, correspondingly more than 2 mg. are used for each cc. of plasma, or the digestion period is prolonged.

[11] In place of weighing out 2 mg. of solid urease one may add 0.20 cc. of a 1 per cent solution of the urease. In this case the subsequent precipitation of proteins is carried out by adding, for each cc. of plasma or diluted blood, 4.8 cc. of picric-citric acid solution from a burette, instead of 5 cc. Unless canavanine-free urease (11) is used, the amount added to the blood must be measured accurately because of its appreciable effect on the blank. The canavanine contributed by 2 mg. of ordinary urease to 1 cc. of plasma may be enough to raise the plasma α-amino nitrogen by 10 per cent.

Hamilton (11) jack bean urease prepared by the acetone precipitation method of Van Slyke and Cullen (8) contains 6 to 8 per cent of the amino acid, canavanine, which evolves CO_2 as do other α-amino acids when heated

TABLE I

Factors by Which P_{CO_2} Is Multiplied to Obtain Mg. of α-Amino Nitrogen per 100 Cc. of Plasma, Blood, or Cells

The 5 cc. portions of filtrate analyzed represent 5/6 cc. of plasma, 5/12 cc. of whole blood, 5/18 cc. of cells. $S = 3.00$ cc. in all analyses. $i = 1.006$ when $a = 0.5$; 1.003 when $a = 2.0$*

Temperature	Plasma		Whole blood		Cells	
	$a = 0.5$	$a = 2.0$	$a = 0.5$	$a = 2.0$	$a = 0.5$	$a = 2.0$
°C.						
15	0.03350	0.1924	0.0965	0.3847	0.1447	0.577
16	37	16	61	32	41	75
17	23	09	57	16	35	72
18	08	01	53	02	29	70
19	0.03295	0.1894	49	0.3787	23	68
20	82	86	45	71	17	66
21	68	78	41	56	11	63
22	55	70	37	41	06	61
23	42	63	34	25	01	59
24	29	56	30	10	0.1395	57
25	17	49	27	0.3696	90	54
26	05	41	23	82	85	52
27	0.03190	34	19	68	79	50
28	80	27	16	54	74	48
29	68	20	12	40	69	46
30	56	13	09	26	63	44
31	45	06	05	12	58	42
32	33	0.1800	02	0.3598	53	40
33	21	93	0.0899	85	48	38
34	09	86	95	71	43	36
35	0.03098	79	92	57	38	34

* S indicates cc. of solution in the gas chamber from which the CO_2 is extracted, and a the volume of the extracted CO_2 gas when its pressure is measured. i is a factor correcting for reabsorption of CO_2 which occurs when its volume is diminished from 47 cc. to 0.5 or 2.0 cc., over saturated acid NaCl solution (3).

with ninhydrin. If the urease is freed of canavanine by dialysis, as described by Archibald and Hamilton (11), it does not increase the blank.

Except for the incubation with urease, and the addition of citric acid

to the picric acid precipitant, the analysis is carried through in all details as described for blood of normal urea content.

The use of urease, instead of the extra 100 mg. of ninhydrin required by Procedure A, saves several cents in each analysis. Procedure B, with purified urease (11) is therefore economical when large numbers of accurate α-amino nitrogen values are required.

C. Urea Is Determined in Separate Analyses and Correction for It Is Applied—The α-amino nitrogen is determined as described for blood of normal urea content, and from the α-amino nitrogen found 0.007 of the blood urea nitrogen is subtracted. This procedure is convenient if blood urea is determined for other purposes. Otherwise Procedure A or B is simpler.

Calculations. The pressure, P_{CO_2}, of CO_2 from amino acid carboxyl groups, is calculated as:

$$P_{CO_2} = p_1 - p_2 - c$$

The α-amino nitrogen per 100 cc. of plasma, whole blood, or cells is calculated as:

$$Mg.\ \alpha\text{-amino N per 100 cc.} = P_{CO_2} \times factor$$

The values of the factors are given in Table I.

To calculate millimoles of amino acids per liter of plasma or blood, use the factors in Table I divided by 1.4.

Derivation of Factors—The factors in the last column of Table I of MacFadyen (3), which give mg. of α-amino nitrogen per 1 mm. of P_{CO_2} when the NaCl-containing reagents are used, are multiplied by 100/(cc. of blood, plasma, or cells represented in the filtrate aliquot analyzed) in order to obtain the factors in Table I of the present paper.

EXPERIMENTAL

Effects of pH and Ninhydrin Concentration on Time Required at 100° to Complete the Reaction with Alanine—The rate of the ninhydrin reaction with amino acids has been shown to increase with the concentration of ninhydrin and with the pH of the solution (2). To determine the time required for complete reaction with varying concentrations of ninhydrin in the presence of picric acid weighed amounts of α-alanine, approximately 0.8 mg. per cc., were dissolved in aqueous 0.5 per cent picric acid[12] and adjusted to the desired pH (glass electrode) by the addition of NaOH. 5 cc. portions were used for reaction at 100° with ninhydrin in the all-glass reaction vessel. Since the decomposition of the amino acids in the presence

[11] About 25 mg. of picric acid are precipitated with the proteins contained in 1 cc. of normal plasma. Hence most filtrates have about half the picric acid concentration of the original picric acid solution.

of a large excess of ninhydrin follows the course of a first order reaction, the logarithm of the percentage of alanine which had not yet decomposed plotted against the time of heating at 100° yielded a straight line. For each pH and ninhydrin concentration we have established this line by a series of analyses and extrapolated to the time necessary to decompose 99.9 per cent of the amino acid; this time we have taken as the interval required for completion of the reaction. The relations of the time necessary for 99.9 per cent completion of the reaction to pH, with different
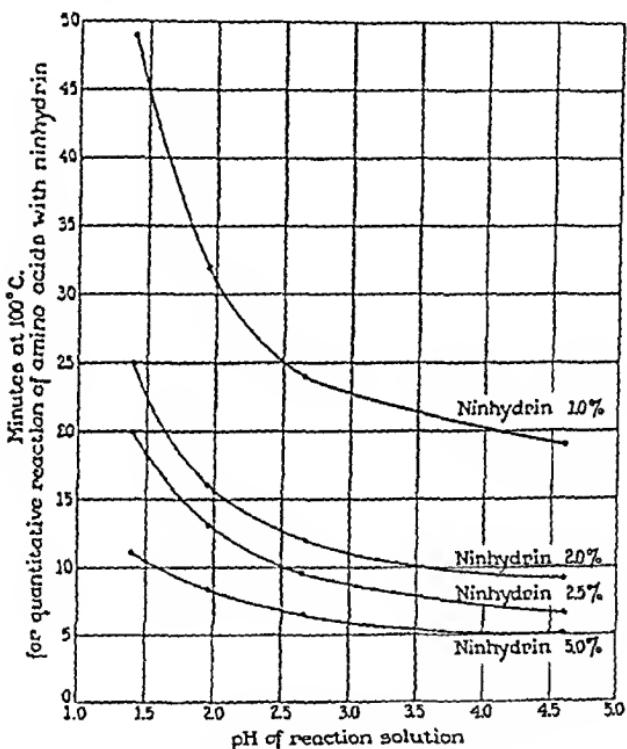
FIG. 2. Effects of ninhydrin concentration and pH on the time required for evolution of 0.999 mole of CO_2 from alanine.

concentrations of ninhydrin, are shown by the curves of Fig. 2. It will be noted that at pH 1.6 or higher, with the 2 per cent concentration of ninhydrin used in analyses of non-uremic blood or plasma filtrates, 20 minutes boiling completes the reaction. It is also obvious that the retarding effect of acidity becomes marked at pH ranges below 2.

Reproducibility of Analytical Values—The upper half of Table II shows plasma α-amino nitrogen concentrations found by analyses of filtrates from separate precipitations of six 1 cc. samples of the same plasma. In the lower half of the table are results obtained by analyses of 5 cc. aliquot

portions of a single filtrate resulting from the precipitation of 5 cc. of plasma with 25 cc. of 1 per cent picric acid. In both instances the mean deviation from the average is only ± 0.3 per cent of the amount measured.

Recovery of Amino Acids Added to Plasma—Alanine and biological mixtures containing amino acids in amounts determined by the ninhydrin-CO_2 method (2) were added to plasma, serum, or whole blood, and the filtrates, obtained after precipitation of the proteins with 1 per cent picric acid, were analyzed. The results are given in Table III.

TABLE II

Reproducibility of α-Amino Nitrogen Values in Protein-Free Filtrates

	Volume of plasma pptd.	Volume of 1 per cent picric acid added to plasma	α-Amino nitrogen of plasma
	cc.	*cc.*	*mg. per 100 cc.*
Analyses of 5 cc. aliquots of filtrates	1	5	6.36
from separate pptns. of 1 cc. portions	1	5	6.39
of same plasma	1	5	6.34
	1	5	6.35
	1	5	6.30
	1	5	6.32
Average... Mean deviation from average, ± 0.02 mg. or $\pm 0.3\%$			6.34
Analysis of 5 cc. aliquot portions of	5	25	6.32
filtrate from pptn. of one 5 cc. por-			6.35
tion of same plasma			6.36
			6.40
			6.34
Average... Mean deviation from average, ± 0.02 mg. or $\pm 0.3\%$			6.35

Behavior of Possible Interfering Filtrate Constituents Other Than Urea—Studies of such substances heated in picric acid solution with and without ninhydrin have indicated the following.

Neither β-hydroxybutyric acid nor glucose breaks down to yield CO_2. Glucose in no way interferes even in concentrations as high as that encountered in uncontrolled diabetes; *e.g.*, 300 mg. of glucose per 100 cc. of plasma.

Acetoacetic acid is so rapidly and completely broken down by heat at pH 2 that the 1 minute boiling of the filtrate before the addition of ninhydrin suffices to eliminate it as a source of CO_2 in the subsequent heating with ninhydrin.

1 cc. of ethyl acetoacetate was treated at room temperature for 24 hours with 20 cc. of 1 N NaOH, by which time the odor of the ester had completely vanished. The solution was warmed slightly and evacuated on the water pump to remove free alcohol. Aliquot portions of the free acid solution were transferred to carboxyl tubes, acidified with 0.1 N HCl to just acid to brom-cresol green indicator. 100 mg. of citrate buffer, pH 2.5, were then added and the volume adjusted to 5 cc. with water. Samples were analyzed with and without boiling the solution for 1 minute as described in the technique of analysis. It was found that 99.8 per cent of the acetoacetic acid was eliminated by the preliminary boiling.

TABLE III

Recovery of Amino Acids Added to Plasma, Serum, and Albumin Solution

Protein solution	Amino acids added	α-Amino nitrogen*				Re-covery
		Plasma (a)	Plasma plus amino acids (b)	In-crease found (b) − (a)	Added	
		mg. per cc.	mg. per cc.	mg.	mg.	per cent
Horse serum	Edestin acid hydrolysate†	0.0612	0.1114	0.0502	0.0504	99.6
		0.0636	0.1088	0.0452	0.0460	98.3
" "	Amino acids of concentrated horse serum filtrate‡	0.0576	0.1051	0.0475	0.0476	99.8
Ox plasma	α-Alanine	0.0389	0.0543	0.0154	0.0156	98.7
Albumin solution (7%)	Pancreatin digest of casein, approximately 50% free amino acids	0.0048	0.0490	0.0442	0.0445	99.3
Human plasma	α-Alanine	0.0437	0.0972	0.0535	0.0530	100.9
" cells	Edestin acid hydrolysate	0.0938	0.2686	0.1748	0.1764	99.2
" "	α-Alanine	0.0903	0.3715	0.2812	0.2804	100.3
" whole blood	"	0.0692	0.3490	0.2798	0.2804	99.7
" " "	Edestin acid hydrolysate	0.0676	0.1854	0.1178	0.1176	100.2

* All analytical values quoted are the average of duplicate determinations.

† Edestin acid hydrolysate. Edestin hydrolyzed with boiling 6 N HCl for 24 hours.

‡ The picric acid filtrate of horse serum had its picric acid content restored to 1 per cent by the addition of solid picric acid to replace that lost by precipitation with proteins. This filtrate was then used to precipitate more horse serum. The amino acids in this filtrate served as a mixture of amino acids identical with that occurring in the plasma.

Pyruvic acid heated under the conditions of the analysis for 20 minutes yields per mole 0.05 mole of CO_2. However, the normal concentration of pyruvic acid in the plasma is only about 1 mg. per 100 cc. of plasma, so that its effect on the analysis would be to increase the α-amino nitrogen measurement by less than 0.01 mg. per 100 cc. of plasma.

MacFadyen, in determining plasma α-amino nitrogen without removing the proteins (3), encountered some plasmas that contained a substance or

substances (other than urea) which spontaneously broke down to give CO_2 on heating to 100° and were eliminated only after the plasma was heated at 100° for 10 minutes. We have encountered such plasmas also but find that in the picric acid filtrates their labile CO_2 is removed by the initial boiling of 1 minute.

α-Amino Nitrogen Concentration of Normal Human and Dog Plasma and Cells—The general range of α-amino nitrogen values for normal human and dog plasma as determined by analysis of picric acid filtrates is given in Table IV. The urea in each case was determined and corrected for by the factor 0.007. The average of 4.07 mg. of α-amino nitrogen per 100 cc. of human plasma is therefore 0.1 mg. per 100 cc. less than the uncorrected mean.

The values yielded by analyses of the picric acid filtrate were compared with those obtained by applying the ninhydrin-CO_2 method in the presence of the plasma proteins as described by MacFadyen (3). In one human plasma (R.) the two methods gave the same result, but in the other twelve the α-amino nitrogen determined in the presence of the proteins was from 0.1 to 0.5 mg. per 100 cc. higher than in the picric acid filtrate. The presumable cause of such a difference is the presence of small but measurable amounts of some substance which has the $\cdot CH(NH_2)\cdot COOH$ group, and is precipitated by picric acid with the proteins. Such a substance might be the phosphatidyl serine discovered in the brain phosphatides by Folch and Schneider (13, 14), but whether it exists in the plasma phosphatides remains to be demonstrated.

From twenty human plasmas, uncorrected for the slight effect of urea, Cramer and Winnick (15) by the present method obtained a mean of 4.2 mg. of α-amino nitrogen per 100 cc. Correction for the urea would reduce this to 4.1 mg., the same as our average value.

If Cramer and Winnick's data and ours are considered together, it appears that the mean normal α-amino nitrogen value of human plasma may be taken as 4.2 mg. per 100 cc. uncorrected for urea, or 4.1 mg. corrected, with the variation ordinarily between 3.4 and 5.5 mg. per cent. Three of Cramer and Winnick's twenty analyses are outside this limit, but, as the subjects were convalescents, it is possible that their condition was not entirely normal.

The results with erythrocytes in Table IV confirm Farr and MacFadyen (16) in showing that the concentration of α-amino nitrogen is about twice as great in the red cells as in plasma; it ranges from 1.7 to 2.2 times as great in the six comparative analyses of Table IV.

Six of the cell and plasma filtrates used for α-amino nitrogen were also used for determination of amino nitrogen by the manometric nitrous acid method of Van Slyke (12). The nitrous acid method would be expected

to give somewhat different results, because, unlike the ninhydrin method, it includes 90 per cent of the *acid amide* nitrogen of glutamine, and NH_2 nitrogen of peptides, amino purines, and other amines outside the class of free amino acids, but does not include two of the amino acids, proline and hydroxyproline. In both cells and plasma the nitrous acid method

TABLE IV

α-Amino Nitrogen and Total Amino Nitrogen Values for Normal Human and Dog Plasmas and Red Blood Cells[*]

The values are given in mg. per 100 cc.

Blood donor	Plasma			Cells	
	α-Amino N		Total amino N	α-Amino N	Total amino N
	Whole plasma	Picric acid filtrate	Picric acid filtrate	Picric acid filtrate	
S.	3.84	3.56	3.78	7.73	10.66
L. N.	4.75	4.48	5.16	9.64	13.13
E.	5.52	5.00			
" (fasting).	4.76	4.32	4.72	7.80	12.54
F.	4.13	3.76	4.26	6.98	9.44
G. (fasting).	3.99	3.67	4.30	7.21	10.64
H.	4.16	3.84	4.19	6.54	8.90
"	4.78	4.45			
" (fasting).	3.85	3.35			
A.	3.92	3.70			
F. P.	4.06	3.90			
R.	4.69	4.72			
B.	4.26	4.15			
Average.	4.36	4.07	4.40	7.65	10.88
Standard deviation.	±0.48	±0.76			
Dog I.	4.20	4.16	5.65		
" II.		3.92	5.65		
" III.		5.50	8.52		
" IV.		4.18	4.94		

* All values were determined in duplicate and corrected for urea. "Total amino nitrogen" values are by the nitrous acid method (12).

gave higher values, the difference averaging 8 per cent of the α-amino N values for plasma, and 42 per cent of the α-amino N values for cells.

Influence of Different Protein Precipitants on α-Amino Nitrogen Values of Plasma Filtrates—From aliquot portions of pooled dog plasma the protein was removed by the different precipitating agents indicated in Table V. α-Amino nitrogen estimations were carried out in duplicate on the filtrates. The results are shown in Table V. It is seen that zinc,

cadmium, and ferric hydroxide all yield values lower than those obtained from the tungstic acid and picric acid filtrates.

The difference between values obtained by precipitation of proteins by aqueous solution of picric acid and those obtained by saturating a protein solution with solid picric acid is marked. It has been found consistently that the precipitation of proteins by first diluting with 5 volumes of water and then saturating with excess of solid picric acid yields filtrates with a lower α-amino nitrogen content than that of filtrates obtained by mixing plasma with 5 volumes of solution of predissolved picric acid. There is

TABLE V

Influence of Protein Precipitants on α-Amino Nitrogen Values of Plasma, Cells, and Whole Blood

Protein solution	Means for removal of proteins	α-Amino nitrogen
		mg. N per 100 cc. plasma
Plasma (dog)	ZnSO₄ + NaOH (Somogyi (17))	2.44
	CdSO₄ + " (Fujita and Iwatake (18))	2.27
	Colloidal ferric hydroxide*	2.98
	Tungstic acid (Folin-Wu (19))	3.33
	" " (Van Slyke and Hawkins (20))	3.49
	Picric acid (present paper)	3.46
	Dialysis†	3.49
Whole blood (human)	Picric acid, aqueous	6.92
	" " solid	5.86
Red blood cells (human)	" " aqueous	9.03
	" " solid	7.10

* Precipitation with ferric hydroxide at room temperature is according to Folch (personal communication). 1 volume of plasma, 8 volumes of water, 1.25 volumes of 5 per cent Fe(OH)₃, and 0.03 volume of MgSO₄ solution (1 gm. MgSO₄·7H₂O:1 cc. water) were brought to 10 volumes with water in a volumetric flask. No heat was applied.

† Hamilton, P. B., and Archibald, R. M., to be published.

apparent adsorption of part of the amino acids by the undissolved picric acid.

Titration Methods for Ninhydrin-CO₂ Reaction—The titration method of Van Slyke, MacFadyen, and Hamilton (21), in which the CO_2 and water of the reacting solution are distilled into standard barium hydroxide, cannot be applied to blood filtrates. Traces of volatile acids distil with the CO_2 and cause positive errors. If picric acid filtrates are used, some of the picric acid also distils over. In Christensen, West, and Dimick's titrimetric application of the ninhydrin-CO₂ method (22) the evolved CO_2 is bubbled through water before reaching the barium hydroxide. This procedure

prevents the error from volatile acids, but the method does not appear to be adapted to precise results with the small amount of amino acids in 1 cc. of plasma: we did not succeed in reducing the error below ± 10 per cent of the amounts determined in the amount of filtrate used for the gasometric procedure.

SUMMARY

The method of Van Slyke, Dillon, MacFadyen, and Hamilton (2) for determination of free α-amino acids, by measurement of the CO_2 evolved from their carboxyl groups by reaction with ninhydrin, has been applied to protein-free filtrates of blood, erythrocytes, and plasma.

Picric and tungstic acids have been shown to yield filtrates without loss of amino acids, but deproteinization with zinc, cadmium, or ferric hydroxide results in losses.

Refinements have been added to the micro manometric technique, so that the 40 or 50 γ of amino acid nitrogen in the filtrate from 1 cc. of plasma can be measured within ± 1 per cent.

A series of normal human plasmas showed amino acid α-nitrogen contents ranging from 3.4 to 5.5 mg. per 100 cc., with a mean of 4.1 mg. For cells the values ranged from 1.7 to 2.2 times as high as for plasma.

The nitrous acid manometric method (12) for determination of primary aliphatic amines showed in plasma mean total amino nitrogen 1.08 times the amounts determined by the more specific ninhydrin-CO_2 method.   In blood cells the nitrous acid method showed an average of 1.42 times as much as the ninhydrin, indicating that erythrocytes contain considerable amounts of amino nitrogen in forms other than the α-nitrogen of free amino acids.

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THE GASOMETRIC DETERMINATION OF AMINO ACIDS IN URINE BY THE NINHYDRIN-CARBON DIOXIDE METHOD

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In this paper the specific ninhydrin-CO_2 method for free α-amino acids (1-3) is adapted to analysis of urine.

Urea is the only interfering substance which appears to be present in urine in significant amounts. Urea hydrolyzes spontaneously to CO_2 and NH_3 when heated to 100° in water solution, about 1 per cent of the urea being hydrolyzed in 5 minutes at 100° between pH 2 and 5 (1, 3). Ninhydrin does not cause nor accelerate the hydrolysis; on the contrary, ninhydrin and urea when warmed in water solution combine to form a compound (4) which resists hydrolysis at 100°, so that urea evolves less CO_2 when heated in the presence of ninhydrin than in its absence. A sufficient excess of ninhydrin is in fact able to abolish almost completely the CO_2 formation from urea (4). However, the amount of urea in urine is so great that to combine it all in the ratio of 3 gm. of ninhydrin to 1 of urea would require expensive amounts of ninhydrin. It is more economical to remove the urea by preliminary hydrolysis with urease. As it is unnecessary to remove the ammonia formed from the urea, the treatment with urease adds little to the manipulations required.

APPARATUS

The apparatus required is that described for the ninhydrin-CO_2 method (1, 3), including the Van Slyke-Neill manometric apparatus (5), water bath, reaction vessels, and calibrated glass spoons.

Of the glass spoons two are required, one to deliver 100 ± 10 mg. of citrate buffer and one to deliver 170 ± 15 mg. of phosphate buffer.

The type of all-glass reaction vessel described for blood filtrate analyses by Hamilton and Van Slyke (3) is the most desirable, although other types can be used (1, 2).

Reagents—Urease. Jack bean urease prepared by the method of Van Slyke and Cullen (6), *viz.* acetone precipitation of an aqueous extract of the beans, is used to remove urea from the urine.[1] As prepared by the acetone

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[1] Urease prepared from jack beans by the method of Van Slyke and Cullen is sold by E. R. Squibb and Sons as Double Strength urease. Squibb's urease not thus designated is mixed with inert material.

method it contains enough amino acid in the form of canavanine to necessitate a correction equal to several per cent of the usual α-amino nitrogen content of the urine.

About two-thirds of the amino acid nitrogen of the acetone-precipitated urease can be removed by washing with alcohol as follows: 5 gm. of the urease are ground in a mortar with ten successive portions of 50 cc. each of 80 per cent ethyl alcohol. The solid residue of about 2.5 gm. is dried at room temperature *in vacuo*.[2]

The amino acid can be entirely removed from the urease by the dialysis purification of Archibald and Hamilton (7).

For use in the analyses, a 10 per cent solution of the urease, crude or purified, is prepared in water, and a portion is diluted 10-fold to make the 1 per cent solution employed.

The *activity of the urease* should be determined as described previously (8) ((9) p. 377). In the activity determination one precaution (Van Slyke and Archibald, unpublished data) should be added to prevent partial inactivation of the dilute urease solution during the determination. Unless 1 per cent or more of protein is present, partial inactivation of the urease may occur during the determination. The inactivation appears to be partly the effect of the high concentration of urea used and partly the effect of traces of dissolved mercury salts in the manometric chamber. To avoid low results from these sources, clean mercury is used in the manometric apparatus, and the chamber is washed with 1 per cent egg albumin immediately before the determination, the mercury in the chamber being thoroughly shaken with the albumin solution. Also, the urease is prepared in 0.1 per cent solution in 5 per cent egg albumin solution. In the determination 2 cc. of the 0.5 M phosphate-1 M urea solution are measured into the albumin-washed chamber of the manometric apparatus, followed by 1 cc. of the albumin-containing 0.1 per cent urease solution, which is measured from a rubber-ringed, stop-cock pipette ((5) p. 532). The time is noted, and the rest of the determination is carried out as previously outlined (8, 9).

If the urease splits less than 0.1 of its weight of urea per minute, the concentration of the urease solution used in the present urine analyses should be increased proportionally above 1 per cent.

[2] When a sample of Squibb's Double Strength urease was thus washed with alcohol, the α-amino nitrogen content was diminished from 6 mg. per gm. of dry urease to 2 mg. If the unwashed urease were used as directed, I mg. per cc. of urine, the α-amino nitrogen added would be 6 mg. per liter of urine, which is 3 to 6 per cent of the amount usually present in the urine (see Table III). The correction for the α-amino nitrogen in the urease is accurately determinable in the blank analysis of the reagents, so that the crude acetone-precipitated urease can be used without error if the amount added to each urine sample is measured. With the washed urease the correction would be only 2 mg. of α-amino nitrogen per liter of urine, and with dialyzed urease (6) the correction is negligible.

Ninhydrin (1, 3).

Approximately 0.5 N NaOH nearly saturated with NaCl. Made by dilution of concentrated (18 to 20 N), carbonate-free NaOH solution with 25 weight per cent CO_2-free NaCl solution (2, 3).

Approximately 2 N lactic acid nearly saturated with NaCl. Made by dilution of 2 volumes of concentrated lactic acid to 10 volumes with CO_2-free 25 weight per cent NaCl solution (2, 3).

Acid and alkali solutions. Approximately 1 N NaOH, 5 N NaOH, 1 N H_2SO_4, and 5 N H_2SO_4.

Indicator solutions. 0.04 per cent water solution of *brom-phenol blue* and 0.04 per cent solution of *brom-cresol green* (sodium salts of both indicators) (10).

Solid citrate buffer for pH 2.5. 2.06 gm. of $Na_3C_6H_5O_7 \cdot 2H_2O$ and 19.15 gm. of $C_6H_8O_7 \cdot H_2O$ ground separately and then together (1).

Solid phosphate buffer for pH 6.2. 3 parts by weight of KH_2PO_4 (anhydrous) and 1 part of Na_2HPO_4 (anhydrous), or 2.5 parts of $Na_2HPO_4 \cdot 12H_2O$. The primary and secondary phosphates are ground separately and then together.

Thymol. Solid, for use as bacteriostatic agent in preservation of urine, and during incubation of urine with urease.

Procedure

Preservation of Urine—If analysis cannot be started within an hour or two after the urine is collected, it is saturated with thymol and stored in a refrigerator. We have kept urine 5 months at 4° saturated with thymol without change in the α-amino nitrogen[2] content.

Removal of Urea with Urease—Place 2 cc. of urine in a ninhydrin reaction vessel, preferably the all-glass, and add 1 drop of 0.04 per cent bromthymol blue. If the reaction is alkaline (blue), add 1 N sulfuric acid drop by drop till the solution is yellow, then 1 N NaOH till just blue (pH a little over 6). If the urine is so acid that the indicator is yellow, add 1 N NaOH until it is just blue. When the reaction is thus adjusted, add 175 mg. of phosphate buffer of pH 6.2, 0.2 cc. of 1 per cent urease solution, and a crystal of thymol. Stopper the vessel loosely, to retard evaporation of water but not the escape of CO_2, and incubate overnight at 37–40°.[3]

Removal of CO_2—After incubation add 1 drop of 0.04 per cent bromcresol green and a drop of capryl alcohol as antifoam. Cautiously add 5 N sulfuric acid until the solution is just yellow (pH approximately 3), then 100 mg. of citrate buffer of pH 2.5. The addition of the sulfuric acid will

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[3] If a reaction vessel with a rubber-connected adapter is used, the adapter should not be used to stopper the vessel during the incubation, as the pores of the rubber would take up so much CO_2 as to make the adapter unusable.

cause frothing owing to the amounts of CO_2 liberated from the ammonium carbonate formed from the urea. The frothing can be controlled by gently whirling the fluid in the reaction vessel. To the acidified solution add two or more alundum pieces to prevent bumping, and boil for exactly 1 minute over a micro burner to expel the CO_2.

Boiling at pH 2.5 causes a slight increase in the free amino acids from hydrolysis of conjugated amino acids present in the urine. The effect is of the order of magnitude to increase the α-amino nitrogen by about 1 per cent for each minute of boiling. Because of this effect, it is desirable to limit the boiling to 1 minute, which is more than enough to remove all CO_2.

TABLE I

Factors for Use with Lactate-Sodium Chloride Solutions for Calculating Mg. of
α-Amino Nitrogen per Liter of Urine

The urine sample is 2 cc.

Temperature	α-Amino nitrogen		Temperature	α-Amino nitrogen	
	$a = 2.0$	$a = 0.5$		$a = 2.0$	$a = 0.5$
°C.			°C.		
15	0.802	0.2010	25	0.770	0.1930
16	0.798	02	26	67	23
17	95	0.1994	27	64	16
18	92	85	28	62	08
19	89	77	29	58	01
20	86	69	30	56	0.1894
21	82	61	31	52	86
22	80	53	32	50	79
23	76	45	33	47	72
24	73	38	34	44	66

Reaction with Ninhydrin—The urine sample prepared as above is cooled below 25° and 100 mg. of ninhydrin are added. The vessel is at once closed and evacuated, and is heated in the boiling water bath for 8 minutes (see (1) pp. 640–643). A shorter boiling time is used for urine than for blood filtrates, because the greater ninhydrin concentration and higher pH in the urine analysis accelerate the reaction.

Determination of CO_2 Evolved from Amino Acids—The CO_2 is transferred to the Van Slyke-Neill chamber and the manometer readings p_1 and p_2 are taken, as described for analyses of blood filtrates (3).

Blank Analysis—A blank analysis on the reagents is performed in which 2 cc. of water are treated with urease, etc., in place of 2 cc. of urine. The $p_1 - p_2$ reading observed in the blank analysis is the c correction.

Calculation. The pressure, P_{CO_2}, of CO_2 evolved from the carboxyl groups of the amino acids is:

$$P_{CO_2} = p_1 - p_2 - c, \text{ and}$$
$$Mg. \; \alpha\text{-}amino \; nitrogen \; per \; liter \; urine = P_{CO_2} \times factor$$

The factors for use when 2 cc. samples of urine are analyzed are given in Table I. They have been calculated from those given by MacFadyen (2) for use when the solution from which the CO_2 is extracted in the Van Slyke-Neill chamber is saturated with NaCl.

EXPERIMENTAL

Recovery of Amino Acids Added to Urine—The results are shown in Table II. In the tryptic digest used casein was approximately two-thirds

TABLE II

Recovery of α-Amino Nitrogen of Alanine and of Tryptic Digest of Casein Added to Urine

Urine No.	α-N of urine alone (a)	Amino acids added		α-N found in urine plus added material (c)	α-N of added amino acids recovered	
		Substance added	α-N added (b)		Mean (c) − (a)	$\frac{100 \, (c - a)}{(b)}$
	mg. per l.		*mg. per l.*	*mg. per l.*	*mg. per l.*	*per cent*
1	139.6	Hydrolysate	237.9	379.8	237.1	100.0
	140.6		237.9	375.7		
2	156.4	"	237.9	389.8	234.3	98.5
	154.1		237.9	389.4		
3	48.5	Alanine	31.3	79.7	31.2	99.8
4	7.5	"	19.3	26.7	19.2	99.4

digested to the stage of free amino acids. The α-amino nitrogen of the digest content was determined as described by Van Slyke, Dillon, Mac-Fadyen, and Hamilton (1) with maximal precision by reaction with ninhydrin at pH 2.5, samples to give over 400 mm. of CO_2 pressure at 2 cc. of gas volume being used. The alanine used was a preparation of purity tested by analysis, and the solutions of it were made up by weight.

Effect on α-Amino Nitrogen Results of Alkaline Treatment to Distil Off Ammonia—In Table III are given results, in the first column, obtained with the ninhydrin-CO_2 method as described in this paper. In the preliminary treatment the urea was hydrolyzed with urease, buffer to keep the pH below 8 being used, the digest was acidified to pH 3, the CO_2 boiled off, and the α-amino nitrogen determined in the acid residue.

In the analyses reported in the second column portions of the same

urease-digested urines were treated with NaOH till the pH was between 10 and 11. The ammonia was then removed by distillation at about 15 mm. pressure, and about 25° temperature. The residue was then acidified to pH 3, and the rest of the analysis completed as usual.

The results show that the alkaline treatment involved in the ammonia removal resulted in a decrease of 3 to 8 per cent in the α-amino nitrogen determined. Presumably the effect was due to the temporary exposure to pH as high as 10 to 11, because the distillation was accomplished without warming above room temperature.

Recovery experiments in which known amounts of amino acids were added to urine and the α-amino nitrogen was determined after the above treatment, with distillation of ammonia, showed that some of the added α-amino nitrogen of these amino acids disappeared during the distillation.

A small fraction of the amino acids apparently reacts with some constituent of the urease-treated urine during the ammonia distillation, and the

TABLE III

Effect of Distilling Off NH_3 at pH 10 to 11 in Vacuo on α-Amino Nitrogen of Urease-Digested Urine

Urine No.	α-N found without removal of NH₃ (regular method described in this paper)	α-N found after distilling off NH₃
1	9.00	9.00
2	17.67	16.54
3	17.35	16.83
4	13.94	13.03

affected amino acids are so altered that they no longer evolve CO_2 when heated with ninhydrin.

The reaction apparently affects the carboxyl groups of the amino acids, and not the NH_2 groups, for recovery of the NH_2 nitrogen of added amino acids was quantitative when determined by the nitrous acid reaction (11), and was not diminished by the treatment involved in the removal of ammonia.

Comparison with Other Methods for Determination of Amino Acids in Urine—For this comparison were chosen the Sørensen "formol" titration (12) as used by Northrop (13) and applied to urine by Van Slyke and Kirk (14) ((9) p. 927), and the manometric nitrous acid method as applied to urine by Van Slyke and Kirk (14) ((9) p. 927). The formol titration and the nitrous acid method have been previously compared by Van Slyke and Kirk (14) ((9) p. 927), who concluded that, in analyses of blood and urine, results obtained with formaldehyde titration were in as good agreement with the nitrous acid method as differences in specificity would warrant.

The formaldehyde titration was applied as described by Van Slyke and Kirk (14) ((9) p. 927) except that the starting point of the titration at pH 7 and the end-point at pH 9 in the present analysis were determined with the glass electrode of MacInnes and Longsworth (15) instead of with indicators. The indicators, litmus for pH 7 and thymolphthalein for pH 9, were used as described by Van Slyke and Kirk, but the pH was then checked with the glass electrode, and if it was not precisely at pH 7 or 9, 0.02 N hydrochloric acid or sodium hydroxide was added to make it exact.

The nitrous acid method was applied throughout as described by Van Slyke and Kirk (14) ((9) p. 927).

Unlike the ninhydrin-CO_2 method, the nitrous acid method and formol titration include not only the α-nitrogen of free amino acids, but also the terminal NH_2 groups of peptides, and the NH_2 groups of purines, pyri-

TABLE IV

Comparison of Results from Different Methods for Determination of Amino Acids in Urine

Method	Gasometric ninhydrin-CO_2	Gasometric nitrous acid, Van Slyke and Kirk (14) ((9) p. 927)	Northrop (13) modification of formaldehyde titration of Sørensen, as applied by Van Slyke and Kirk (14) ((9) (p. 927)
Preliminary treatment of urine before measurement of α-amino N	Urea hydrolyzed with urease; CO_2 boiled off	Urea hydrolyzed with urease; NH_3 distilled off *in vacuo*	NH_3 distilled off *in vacuo*
	mg. α-N per l.	*mg. amino N per l.*	*mg. amino N per l.*
Urine 1	98	118	127
" 2	177	176	254
" 3	174	236	330
" 4	139	168	191

midines, and aliphatic amines other than amino acids. In the formol titration such amines are completely included, while in the nitrous acid method they are only partially included, because NH_2 in most other amines reacts more slowly than in the α-amino acids. As might be expected from these facts, the nitrous acid method shows in Table IV amino nitrogen values ranging upwards from the α-nitrogen values, and the formol titration shows still higher values. The margins by which the results of the formol and nitrous acid methods exceed the α-nitrogen values are presumably indications of the proportion of peptides and amines present in addition to the amino acids.

SUMMARY

The gasometric determination of free amino acids by the specific reaction with ninhydrin to evolve CO_2 has been applied to urine.

As might be expected from the greater specificity of the ninhydrin-CO_2 reaction, the method gives somewhat smaller values than the nitrous acid method or the formaldehyde titration method, both of which measure other amines as well as amino acids.

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AMINO ACID NITROGEN OF NORMAL HUMAN PLASMA*

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The two principal methods which have been employed for the determination of the amino acid concentration of blood are those of Van Slyke (1) and Folin (2). In the first method the nitrogen which is evolved upon treatment of the protein-free blood filtrate with nitrous acid, following destruction of the urea with urease, is measured. The second procedure depends upon the development of a red color with β-naphthoquinonesulfonic acid and alkali.

Recently Van Slyke, Dillon, MacFadyen, and Hamilton (3) have described a quantitative method for free amino acids, in which carbon dioxide, evolved from the carboxyl groups by reaction with ninhydrin, is measured in the Van Slyke-Neill manometric apparatus. This procedure is free from certain of the difficulties inherent in the previous amino acid methods, and is specific for free α-amino acids.

In the present study this manometric ninhydrin method was applied to the determination of the amino acids of human blood plasma. Results are reported for a series of twenty normal adult individuals.

EXPERIMENTAL

Oxalated blood was taken from ten male and ten female adult convalescent patients of the Detroit Receiving Hospital. The plasma non-protein nitrogen and urea levels were normal in these individuals. The blood was drawn after a 12 hour fast in every case, and centrifuged at once. The amino acid carboxyl carbon dioxide was determined in picric acid filtrates of the plasma, as described by Hamilton and Van Slyke (4). Their reaction vessel with a mercury layer to prevent contact with rubber was employed.

Non-protein nitrogen and urea nitrogen determinations were made also on portions of the plasma. The former was determined on trichloroacetic acid filtrates by the micro-Kjeldahl method, while urea was measured by the Conway micro diffusion method (5).

The results obtained are summarized in Table I. The amino acid values reported are the averages of duplicate determinations. The average deviation between these duplicate analyses was 3.3 per cent.

The average values for α-amino nitrogen are 4.3 and 4.1 mg. per 100 ml. of plasma for males and females respectively. This difference is not significant. The α-amino nitrogen constitutes 16.5 per cent and the urea nitrogen 49.5 per cent of the average non-protein nitrogen value for both sexes.

TABLE I

α-Nitrogen of Amino Acids of Normal Plasma

Males			Females		
α-Amino nitrogen	Urea nitrogen	Non-protein nitrogen	α-Amino nitrogen	Urea nitrogen	Non-protein nitrogen
mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*	*mg. per 100 ml.*
3.7	10.5	20.7	3.6	9.7	23.6
4.9	14.7	31.2	3.6	10.5	23.7
4.5	18.1	38.3	4.4	21.4	36.4
4.6	13.7	29.6	3.4	13.3	27.0
4.4	13.1	25.5	4.3	13.2	29.5
4.6	12.1	23.5	3.4	10.1	18.8
3.5	14.8	29.4	2.7	6.7	14.2
2.3	11.4	20.6	3.0	9.0	17.4
4.0	12.4	23.8	5.0	13.4	27.9
6.6	10.5	21.5	7.3	15.2	31.0
Mean values 4.3	13.1	26.4	4.1	12.3	25.0
Standard deviations 1.0	2.1	6.6	1.2	3.8	6.4

SUMMARY

The gasometric ninhydrin-CO_2 method was applied to the determination of the free amino acids of normal human plasma.

The average value for a series of ten male and ten female subjects was 4.2 mg. of α-amino nitrogen per 100 ml. of plasma.

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LETTERS TO THE EDITORS

ON THE NATURE OF SERINE DEHYDRASE AND CYSTEINE DESULFURASE

Sirs:

In view of a recent publication[1] in which evidence for the dehydrase nature of the deamination of serine by *Escherichia coli* was presented, it is considered desirable to report certain observations which may bear on this question. It has been reported that *Escherichia coli*[2] and livers of various mammals[3] contain enzymes which convert cysteine to hydrogen sulfide (desulfurase activity). In the present investigation it has been found that brewers' yeast and bakers' yeast also contain cysteine desulfurase systems. Cell-free solutions of the enzymes were prepared from the microorganisms by the procedure of Werkman *et al.*[4] When the solutions were dialyzed against water, the desulfurase activity decreased markedly but was restored by the addition of Zn, Mg, or Mn ions to make a final concentration of 0.001 M.

Crude extracts or reactivated dialyzed extracts representing 10 mg. (dry weight) of *Escherichia coli* or brewers' yeast were found capable of converting 80 to 90 per cent of 4 mg. of cysteine to hydrogen sulfide and ammonia in 24 hours. The addition of 20 mg. of glucose, serine, or phosphoglyceric acid completely inhibited the desulfurase activity of the crude extracts. Serine and phosphoglyceric acid, but not glucose, inhibited the desulfurase activity of the reactivated dialyzed extracts. It is suggested that the inhibition is of a competitive nature, since the extracts were found to attack serine with a formation of ammonia and to attack phosphoglyceric acid with a formation of phosphopyruvic acid (enolase activity).[5] In addition, it was found that the inhibition disappears during prolonged digestion and is proportional to the amount of inhibiting substrate in the digest.

The enzyme attacking serine was found to be inactivated by dialysis

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and to be reactivated by the addition of inorganic ions in the same manner as for the enzyme attacking cysteine and similar to the reported behavior of enolase.[5] The addition of 20 mg. of cysteine or serine completely inhibited the breakdown of 4 mg. of phosphoglyceric acid by the reactivated dialyzed extracts. 0.001 M fluoride inhibited the action of the dialyzed reactivated extracts on serine and cysteine in a manner identical with that reported for enolase.[5] 0.001 M cyanide inactivated the serine enzyme only when reactivation had been effected with Zn ions but inactivated the cysteine enzyme reactivated with Zn, Mg, or Mn ions.

The desulfurase and serine dehydrase of mammalian tissue were found to be similar to those of the microorganisms. The desulfurase activity of an extract of mouse liver was lost during dialysis and was regenerated by the addition of Zn, Mg, or Mn ions. The dialyzed extract, reactivated with Mg, attacked serine and phophoglyceric acid with the formation of ammonia and phosphopyruvic acid respectively. Desulfurase activity could not be demonstrated in crude extracts of mouse muscle, but dialyzed extracts, to which Mg ions had been added, attacked cysteine with a formation of hydrogen sulfide and ammonia and attacked serine with a formation of ammonia.

All digestions were conducted in 0.06 M bicarbonate in evacuated Thunberg vessels at 37°. The digestions with the reactivated muscle or liver extracts were carried out for a period of 8 hours, during which extracts containing 10 mg. of material (dry weight) effected a 20 to 30 per cent destruction of the cysteine or serine. Pyruvic acid, as measured colorimetrically by the dinitrophenylhydrazone reaction, was a product of the action of all the reactivated dialyzed extracts upon serine or cysteine.

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COMPLEX FORMATION BETWEEN SYNTHETIC
DETERGENTS AND PROTEINS

Sirs:

An investigation has been under way in this laboratory on the application of synthetic detergents to the fractional precipitation, denaturation, and regeneration of globular proteins. In the light of a recent report by Lundgren et al.[1] on the electrophoretic properties of complexes between egg albumin and alkylbenzenesulfonates, formed on the *alkaline* side of the isoelectric point, it may be of interest at this time to point to certain analogies between their results and those which have been obtained independently by us.

We have found that complex formation between detergents and proteins also occurs on the *acid* side of the isoelectric point. Kjeldahl nitrogen analysis indicates that in this pH region crystalline horse serum albumin is completely precipitated by sodium dodecyl sulfate (SDS) within the limits under which the ratio of the weight of protein to detergent varies from 5:1 to 2.5:1. Under these conditions the detergent is completely bound by the protein. Excess of detergent leads to solution of the precipitate. Precipitation appears to be caused by electrostatic forces, since (1) it occurs only on the acid side of the isoelectric point of the protein, the maximum pH approximating the isoelectric point; and (2) the maximal concentration of SDS required for complete precipitation corresponds closely to the total acid-binding capacity of the protein (144 moles of SDS per gm. of protein $\times 10^5$).

Complex formation on the *alkaline* side of the isoelectric point is evidenced by anomalous electrophoresis in the presence of detergent quite analogous to that already reported.[1]

Dissociation of the complex and removal of alkyl sulfate with barium chloride yield electrophoretically homogeneous protein; however, its mobility at pH 7.6, $\mu = 0.1$, is somewhat higher than that of native protein; *i.e.*, 6.70 as compared to 6.25×10^{-5} cm.^{2} sec.$^{-1}$ volt^{-1}. Viscosity measurements indicate that the hydrodynamic volume of the recovered protein is about 25 per cent less than that of the native.

While, for anionic detergents, *precipitation* is confined to the acid side of the isoelectric point, *denaturation*, as revealed by viscosity measurements, occurs in both acid and alkaline regions. Relatively low concentrations of detergents exert a high denaturing action compared to that of urea or guanidine hydrochloride. For example, the intrinsic viscosity of

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serum albumin is increased from 4.3 to 22 by 8 M urea[2] but to 19 by 0.035 M SDS and to 25 by 0.17 M SDS.   In electrophoretic and solubility properties, protein regenerated from regions of detergent excess resembles that recovered from the precipitated protein-detergent complex.

A detailed report on the conditions governing precipitation of proteins by anionic detergents is in preparation.   Further physicochemical and immunochemical investigations on detergent-treated serum proteins are under way.

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THE EFFECTS OF SPERMIDINE AND OTHER POLYAMINES ON THE GROWTH INHIBITION OF ESCHERICHIA COLI BY ATABRINE*

Sirs:

While studying the inhibiting effect of atabrine on the growth of *Escherichia coli*, we have observed that the effect of the drug can be nullified by a substance or substances present in 1 per cent solutions of Witte's peptone.

1 per cent Bacto-peptone, 0.6 per cent NaCl, 0.2 per cent glucose, pH 7.25, inoculated with 1 drop of a 1:1000 dilution of a 24 hour culture of *Escherichia coli* in Bacto-peptone. The amines were adjusted to pH 7.4 before sterilization. Atabrine was sterilized by passage through a Jena sintered glass sterilizing filter. Final concentration of atabrine in all tubes, 0.0005 M. Visible growth in the control tubes in the absence of atabrine occurred in 5 hours. Visible growth in the tubes containing atabrine and medium alone did not occur within 24 hours.

Substance added	Hrs. required for visible growth at		
	0.00025 M	0.0005 M	0.001 M
Spermidine	7	6	6
Spermine	7	10	22
Diethylenetriamine*	>24	20	10
Triethylenetetramine*	23	9	7
Tetraethylenepentamine*	20	$7\frac{1}{2}$	$6\frac{1}{2}$
Ca pantothenate	20	9	$6\frac{1}{2}$

* Commercial samples available to us by the courtesy of the Carbide and Carbon Chemicals Corporation, New York.

Similar substances were present in smaller amount in Mead Johnson's amigen and Bacto-yeast extract and, to a slight extent, in Bacto-technical Casamino acids and Stearns' amino acids.

In examining a large number of pure compounds for similar antagonism to the action of atabrine on the growth of *Escherichia coli*, we obtained positive results with the naturally occurring amines, spermidine and spermine. Three synthetic polyamines of related structure were also active, as was calcium pantothenate, although higher concentrations of these substances were required to show an effect under our experimental conditions. Spermine, at higher concentrations, was apparently toxic. Pertinent data are given in the table.

serum albumin is increased from 4.3 to 22 by 8 M urea[2] but to 19 by 0.035 M SDS and to 25 by 0.17 M SDS.   In electrophoretic and solubility properties, protein regenerated from regions of detergent excess resembles that recovered from the precipitated protein-detergent complex.

A detailed report on the conditions governing precipitation of proteins by anionic detergents is in preparation. Further physicochemical and immunochemical investigations on detergent-treated serum proteins are under way.

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THE PASTEUR EFFECT IN BONE MARROW, STUDIED WITH CARBON MONOXIDE-OXYGEN MIXTURES*

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The recent experiments of Stern and Melnick (1, 2), by the photochemical method, have adduced evidence of the existence of a Pasteur enzyme in retina, yeast, and heart muscle. This they define as the heavy metal-containing thermolabile agent which catalyzes the inhibition of fermentation (or glycolysis) by molecular oxygen, a phenomenon known as the Pasteur reaction. The spectral characteristics of the enzyme in the three tissues studied are not identical, and Stern (3) has pointed out that there may be a number of Pasteur enzymes, each characterized by a specific affinity for oxygen. In each tissue, the spectrum of the Pasteur enzyme closely resembles that of the respiratory enzyme but differs in certain details.

Since bone marrow is one of the few mammalian tissues which responds to lowered oxygen tension by increased activity, Warren (4) studied its respiration and glycolysis at lowered oxygen tensions with the idea of determining whether or not it is necessary to postulate the existence of a Pasteur enzyme in this tissue in order to account for its physiological behavior. It was found that as respiration was decreased by lowering the oxygen tension glycolysis increased in a reciprocal manner, implying that if a Pasteur enzyme is present it has the same affinity for oxygen as the respiratory enzyme (*cf.* (3) p. 100). However, he pointed out that under his experimental conditions, as well as in most manometric experiments, respiration and glycolysis were undoubtedly influenced by diffusion and solubility factors as well as by the level of the oxygen tension. The present paper is an extension of these studies in which, by using carbon monoxide rather than oxygen tension as the variable, changes in respiration and glycolysis are obtained at partial pressures high enough to exclude effects due to diffusion and solubility factors.

Methods

Suspensions of cells of rabbit bone marrow in neutralized serum were prepared as previously described (4). Carbon monoxide-oxygen mixtures were obtained by filling a bottle with CO, reducing the pressure by a known

amount, and then admitting oxygen until atmospheric pressure was again obtained. The accuracy of this method was found to be entirely satisfactory (within ±0.5 per cent) when the gas mixtures were analyzed for oxygen by the dropping mercury electrode method (5). The cell suspensions were placed in small Warburg vessels and equilibrated with the CO-O_2 mixtures while being shaken in the constant temperature bath at 38°. 3 liters of each gas mixture were run through the vessels over a period of 10 to 15 minutes. Control vessels containing serum without cells were included in order to correct for any pressure changes due to CO being absorbed by the alkali in the center wells. Respiration was measured manometrically in the usual way; lactic acid formation was measured chemically by the method of Barker and Summerson (6). In each experiment, respiration and glycolysis were also measured in 100 per cent O_2, and anaerobic glycolysis (the same in 100 per cent CO and 100 per cent N_2) was determined chemically. Duplicate samples of the suspensions were incubated in each gas mixture, usually for 3 hours, and duplicate lactic acid analyses were made on each of the samples. The accuracy of the determinations and the method of calculating the results are the same as previously described (4). In order to obtain hyperplastic marrows satisfactory for preparing cell suspensions, most of the animals were bled several times before being sacrificed; accordingly, erythroid cells predominated in the suspensions.

Results

The results of thirteen experiments are summarized and shown graphically in Fig. 1, in which the results at each partial pressure of CO are averaged. The smooth Curve A is drawn by inspection through the points representing per cent decrease in respiration. It is of interest that the shape of this curve closely resembles that describing the changes in respiration of yeast suspensions in various CO-O_2 mixtures reported by Winzler (7). Curve B, taken from the earlier publication (4), shows the per cent decrease in respiration produced at lowered oxygen tensions. It is clear that the sensitivity of the marrow cells to the effects of carbon monoxide permits most of the measurements to be made at gas tensions at which diffusion cannot be a limiting factor.

The per cent increase in glycolysis is indicated in Fig. 1. The spread of these data is greater than that for changes in respiration, probably because of the difficulty of measuring accurately relatively small changes in lactic acid production in the presence of considerable amounts of lactic acid in the serum. The accuracy of the lactic acid analyses under these conditions is within ±5 per cent, but, at the extremes of the curve, the error in per cent change in glycolysis so introduced is about ±10 per cent. Accordingly, most of the measurements were made in the intermediate zone and it is sig-

nificant that the average figure of four determinations at 92 per cent CO is one of the points falling most nearly on the curve. It appears, in fact, that the per cent increase in glycolysis does not differ significantly from the per cent decrease in respiration, or in other words that the two changes are reciprocally related.
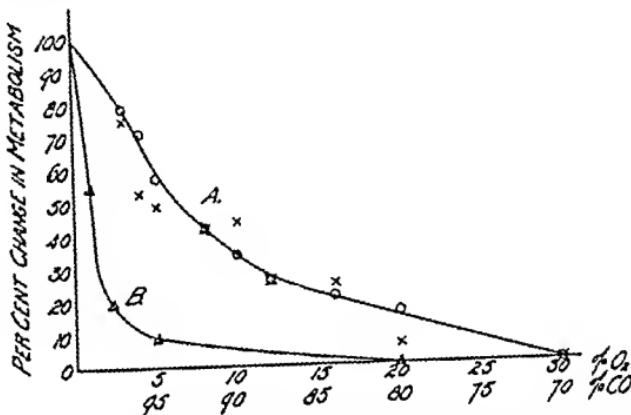
FIG. 1. Respiration and glycolysis of bone marrow suspensions in various $CO-O_2$ mixtures. Curve A, circles indicate per cent decrease in respiration; crosses, per cent increase in glycolysis. Curve B, per cent decrease in respiration at low oxygen tensions (cf. (4)).

DISCUSSION

The reciprocal relationship between the changes in respiration and glycolysis described above implies that, if a Pasteur enzyme is present in rabbit bone marrow, it has the same affinity for CO as the respiratory enzyme. But it has already been shown (4) that these two enzymes have the same affinity for oxygen. Rather than postulate the existence of two enzymes, both having the same affinities for oxygen and CO, it seems preferable to interpret the results as being due to the effects of oxygen lack or CO on a single (respiratory) enzyme. In the light of this evidence, the existence of an independent Pasteur enzyme in bone marrow seems extremely doubtful.

Recently, Craig and Beecher have found a reciprocal relationship between the changes in respiration and glycolysis at low oxygen tensions in brain cortex (8) and rat retina in phosphate medium (9). In bicarbonate medium, the changes in respiration and glycolysis of retina were not reciprocal, confirming Laser (10). In bone marrow, the nature of the medium does not complicate the situation, for the reciprocal relationship is found in serum with or without bicarbonate (4). We concur with the conclusion of Craig and Beecher that oxygen tension may act on respiration and glycolysis through the mediation of a common agent.

SUMMARY

1. The exposure of suspensions of rabbit bone marrow cells to high tensions of carbon monoxide results in a decrease in the rate of respiration accompanied by a reciprocal increase in glycolysis.

2. These results offer no support for the existence of a Pasteur enzyme in this tissue.

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CHEMISTRY OF THE CHICK EMBRYO

IV. AMINOPEPTIDASE[*]

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In a previous report of this series (7) we have demonstrated the quantitative accumulations of wet weight, nitrogen, and dipeptidase by the developing chick embryo. The present communication concerns aminopeptidase and includes comments on the choice of substrate, glycylglycyl-*d*-alanine, its synthesis, the properties of the chick embryo enzyme, and quantitative data on its accumulation during development.

Choice of Substrate—For the quantitative investigation of an enzyme in a tissue extract it is desirable to use a substrate which is subject to a single well defined action in the presence of the multiple enzymes of the extract. The results, then, are more certain than otherwise to give an adequate representation of a single function. Processes intended to inactivate other enzymes or isolate the enzyme partially or completely from others are open to the suspicion that the desired enzyme is also partially inactivated, incompletely isolated or recovered, or removed from essential activators. A properly selected substrate for our purpose should be specific to the enzyme and should be as closely related to the expected natural substrates as possible.

A substrate which fulfils these requirements with respect to aminopeptidase in chick embryo extracts is glycylglycyl-*d*-alanine. It is hydrolyzed to glycine and glycyl-*d*-alanine and the latter is not at all, or only slowly, acted upon by chick embryo extracts. The tripeptide differs from a possible natural substrate in the configuration of the alanine residue only.

Action of Chick Embryo Extract on Glycylglycyl-d-alanine—The hydrolysis of the peptide bond between the two glycyl residues is demonstrated by the following experiment. A 30 per cent glycerol extract of chick embryo was prepared and mixed with 7 c.mm. of substrate solutions at pH 8.4 containing 0.1 M glycylglycyl-*d*-alanine (Substrate A), 0.1 M glycyl-*d*-alanine and 0.1 M glycine (Substrate B), or 0.1 M glycylglycine and 0.1 M *d*(−)-alanine (Substrate C). At intervals, samples were taken and titrated as described below with the results shown in Table I. It is evident that glycylglycine is

rapidly hydrolyzed, that glycylglyeyl-*d*-alanine is more slowly hydrolyzed at one peptide link only, and that glyeyl-*d*-alanine is practically not hydrolyzed. The possible products of hydrolysis at one bond of the tripeptide are contained initially in Substrates B and C. If Substrate C contained the products, then both peptide bonds should have been hydrolyzed in Substrate A. Since only one bond was hydrolyzed, the mixture in Substrate B is the same as the end-products of hydrolysis of Substrate A. Thus, the activity is properly defined as that of an aminopeptidase.

Optimum pH—Substrates containing phosphate buffers and varying in pH were prepared and the extent of hydrolysis over a fixed period was measured. The optimum pH was found to be 8.4 in phosphate buffers and then confirmed with simple mixtures of substrate and NaOH. The substrate used in subsequent work was, therefore, a solution of 0.1 M glycyl-

TABLE I

Position of Hydrolysis of Glycylglycyl-d-alanine by Chick Embryo Extract
Temperature 30°; pH 8.4.

Substrate	Per cent hydrolysis of 1 group after			
	2 hrs.	4 hrs.	24 hrs.	48 hrs.
A. Glycylglycyl-*d*-alanine..................	23	42	92	101
B. Glycyl-*d*-alanine and glycine............	0	0	2	5
C. Glycylglycine and *d*(−)-alanine.........	92	98	100	101

glycyl-*d*-alanine in 0.07 N NaOH. The substrate and products of hydrolysis acted as sufficient buffer for our purposes.

Measurement of Activity of Extracts—It was found that the rate of hydrolysis could not be expressed by any simple kinetic equation, and we therefore resorted to the device which proved useful in our studies of dipeptidase. A single concentrated extract was prepared and its activity determined at a series of dilutions. The activities were determined by mixing 8.1 c.mm. of diluted extract with 7.0 c.mm. of substrate solution containing 0.1 M glycylglycyl-*d*-alanine and 0.07 N NaOH, incubating at 30° for 3 hours, stopping the action by adding 1.2 micromoles of HCl in alcohol, and titrating the mixture in acetone with 0.046 N alcoholic HCl, naphthyl red being used as indicator (9). The extent of hydrolysis is measured by the increased HCl used over a blank to which alcoholic HCl was added at zero time. The extract was diluted with 20 per cent glycerol in order to make the diluted extract comparable to those ordinarily prepared. The standard curve is shown in Fig. 1 along with the experimental points used in establishing it. The validity of this curve as a measure of relative quantities of

enzyme has been checked a number of times by estimating the enzyme in a given extract at two or more dilutions.

A unit of aminopeptidase exhibits the activity shown by 1 c.mm. of the standard extract under the conditions specified. The same standard curve was used throughout.

Preparation of Extracts—The incubation practice and isolation technique have been previously described. Except for the use of a ratio of 1:4 for weight of embryo to volume of extract, the extracts were prepared as for the dipeptidase estimations (12).

Accumulation Data—Determinations were made on embryos at from 1.5 to 18 days incubation. For the smallest embryos it was necessary to combine several embryos to make an adequate amount of extract. About 250 individual embryos were used.
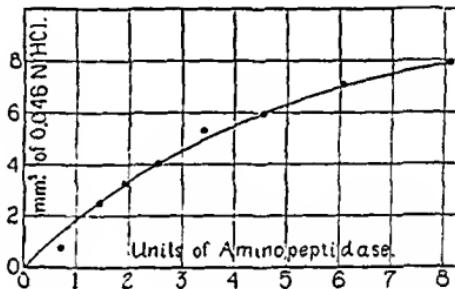
FIG. 1. Standardization curve for aminopeptidase. Substrate, glycylglycyl-*d*-alanine. Temperature 30°. The unit contained in 1 c.mm. of standard extract.

Estimation of Incubation Age—Although it is feasible to record the actual times of incubation of all the embryos used, certain considerations lead us to adopt a calculated incubation age (A_c) as the basis for analysis of the data. There is considerable variation in the weights of embryos of the same incubation age, which may be attributed to variations in the extent of "body heating," slight variations of incubation conditions, and to "biological" variability. The actual state of development of a given embryo is, we believe, more reliably indicated by its weight than by the time which it has been in the incubator. Therefore, the ages of the embryos are calculated from their weights.

The data, consisting of a series of weights of embryos in mg. with the corresponding series of estimations of aminopeptidase in units per mg., were arranged in the order of the weights. The series was then divided into equal groups (eight embryos each), the group averages calculated, and the age corresponding to the average group weight (W) calculated with the weight

accumulation equations developed from previously reported weighings of
many embryos.   The equations used were

$$\log A_c = 0.215 + 0.192 \log W$$

for $P_{4.33}^{(1.5)}$ and

$$\log A_c = 0.009 + 0.286 \log W$$

for $P_{(18.0)}^{4.33}$.   The superscripts and subscripts of P indicate the beginning and
end, respectively, of the period in incubation days for which the equations
are valid.   Enclosure of the superscript or subscript in parentheses indi-
cates the age limit of the data available.
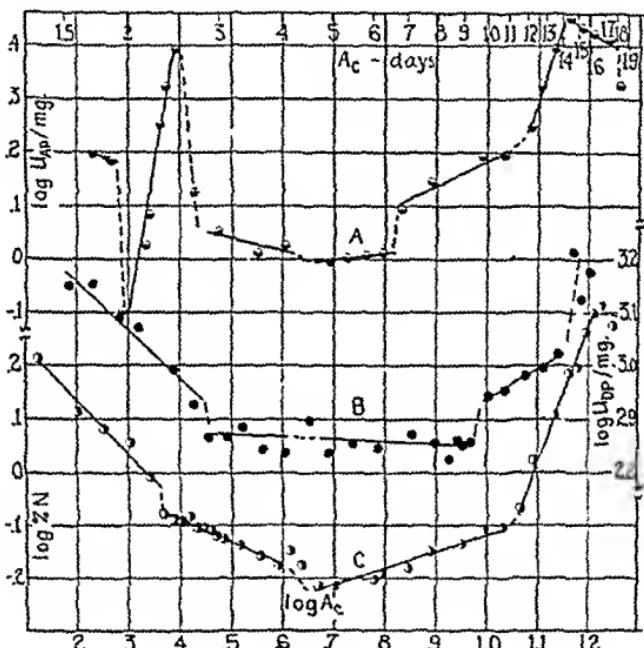
Fig. 2. Relative accumulation diagrams for aminopeptidase (Curve A), dipep-
tidase (Curve B), and N (Curve C).   The dash lines indicate interphases and are
uncertain. U_{AP} represents the number of units of aminopeptidase as defined in the
text and U_{DP} the number of units of dipeptidase as defined by Levy and Palmer (7).

The parameters of these equations were derived from the data reported
by us (7) for eggs of the same breed and source as those used for the present
work.   The parameters were determined by fitting the appropriate data by
the least squares method and so differ slightly from those calculated from
the phase data given by Levy and Palmer (7) in which the fitting was done
with a straight edge on large scale plots.

The calculation of the age from the weight eliminates some of the vari-
ability occurring between embryos of the same incubation time and so

reduces the number of embryos which must be examined to establish the parameters of the growth equations and the interphase times.

Interphases and Parameters of Accumulation—The slopes observed in a plot of log Q (Q represents the quantity of any considered material) against log A (the age in days) are large, and some uncertainty results in the measurement of the slopes (a_Q) and intercepts (i_Q) because it is inconvenient to have the two axes with equal scalar values. By subtracting the equations for weight, log $W = i_W + a_W \log A$ from the general equation for any other quantity Q, log $Q = i_Q + a_Q \log A$, we obtain log $Q/W = (i_Q - i_W) +$

TABLE II

Parameters of Phase Equations

Aminopeptidase

Interphase times, days	(1.5)	1.9	2.6	4.3	6.6	11.9	14.5	(18)
a_Q	4.9	10.7	5.0	3.6	4.0	6.4	2.9	
i_Q	0.9	-2.9	-1.0	-0.1	-0.3	-2.9	-1.1	

Dipeptidase

Interphase times, days	(1.5)		3.6	4.3	9.6	14.8		(18)
a_Q		4.3		5.2	3.45	2.9		?
i_Q		1.2		1.8	2.9	2.4		

Nitrogen

Interphase times, days	(1.5)		2.4	4.3	11.5	16.2		(18)
a_Q		4.2		4.8	3.8	6.0		?
i_Q		-2.9		-3.0	-2.5	-4.8		

The figures in parentheses indicate the ages of the youngest and oldest embryos studied, rather than interphase times.

$(a_Q - a_W) \log A$, which does not have this inconvenience. In order to estimate i_Q and a_Q (i_W and a_W being known) we plot log Q/W; *i.e.*, log concentration against log A. This plot is called a relative accumulation diagram and is shown in Fig. 2 for aminopeptidase (Curve A), for dipeptidase (Curve B), and for nitrogen (Curve C). The data for the latter two have been previously reported, but have now been grouped and treated as indicated above for aminopeptidase. The new values for the interphases and phase parameters are given in Table II.

DISCUSSION

Needham (11) has shown that data on chemical growth of organisms can exhibit the heterogonic relations of Huxley (6). That is, if y represents the

five portions at short intervals along with portions of 2 N NaOH sufficient to keep the solution alkaline to phenolphthalein (used as an outside indicator). After being removed from the ice bath, the mixture was stirred for an hour and the alkaline solution filtered. It was then acidified to Congo red with concentrated HCl and cooled to 5°. The precipitate was recrystallized by dissolving in 100 ml. of boiling absolute alcohol to which 200 ml. of hot water were added. After standing overnight at 5°, 7.2 gm. (55 per cent) of fine needles were collected which melted at 191–192°. These had a titration equivalent of 339 (theory 337) toward NaOH and phenolphthalein. For the estimation of optical activity, the substance was dissolved in an exact equivalent of NaOH solution. $[\alpha]_5^{22} = +10.1°$. Carbobenzoxyglycylglycyl-*dl*-alanine prepared in a similar manner melted at 184–185°.

Glycylglycyl-d-alanine—The carbobenzoxyglycylglycylalanine was hydrogenated with a Pd catalyst, according to Bergmann and Zervas (3). The product was recrystallized several times by dissolving in minimal quantities of hot water and adding alcohol. The yield was 78 per cent of theory. The formol titration equivalent was 201.5 (theory 203). $[\alpha]_5^{20} = +35.6°$ in water. pK_2 of the peptide was found to be 8.04 by a quinhydrone electrode system.

SUMMARY

A tripeptide, glycylglycyl-*d*-alanine, in which the carboxyl group is carried by the residue of an unnatural amino acid is shown to be hydrolyzed by chick embryo extract from the amino end only. Its hydrolysis is used to estimate the aminopeptidase contents of crude whole chick embryo extracts.

The accumulation of aminopeptidase during development of the chick embryo is demonstrated. Certain modifications in the method of plotting growth data are used, particularly the grouping of data by weights of embryos from which the age is calculated and the use of a relative accumulation diagram on which the logarithm of concentration per unit wet weight is plotted against the logarithm of calculated age. These offer certain conveniences and consistencies not otherwise available.

The accumulation of aminopeptidase, like that of all other enzymes and entities previously measured, shows abrupt changes in rate during development. The conditions governing the appearance of "interphases" at which such changes occur are discussed.

The synthesis of glycylglycyl-*d*-alanine is described.

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workers (3), who have studied the spontaneous transformation from a physiological point of view, did not observe the slightest reducibility of the transformation product. Prolonged treatment with hydrogen sulfide and particularly with excessive glutathione did not yield any ascorbic acid. These workers felt, therefore, justified in considering the spontaneous transformation of dehydroascorbic acid proceeding under their experimental conditions (pH 7, phosphate buffer, anaerobically) as an irreversible process.

From the lack of agreement in the observations made by Herbert *et al.* and Borsook *et al.* as regards the reversibility or non-reversibility of the spontaneous transformation, it is doubtful whether the reversible Equation 1 proposed by the Birmingham investigators has any bearing upon the biological and obviously irreversible process studied by the American workers. Hence diketogulonic acid, being itself slowly reducible with hydrogen sulfide, cannot be considered as a possible chemical equivalent of the *irreversible* transformation product of dehydroascorbic acid. Nevertheless, the conception of diketogulonic acid both as a chemical entity and as the product of the biologically irreversible transformation has since found acceptance in the literature (4, 5). In their original paper, Borsook *et al.*, on the other hand, expressly preferred not to make any statement concerning the structure of their transformation product.

From the results of Borsook *et al.* one is inclined to assume the existence of a more degraded and therefore irreversible transformation product. Hence we have attempted in the present work to establish whether in the presence of phosphate (the only additional factor used by Borsook *et al.*) the spontaneous transformation of dehydroascorbic acid leads to an irreversible transformation product not identical with diketogulonic acid. The only assumption made is that the non-oxidative formation of oxalic acid, occurring normally around and above pH 10, may take place in the biological range (pH 7) by the catalytic influence of phosphate. At first sight such action of phosphate seems unfamiliar; it appears, however, less unexpected in view of the positive influence of phosphate upon the evolution of CO_2 during the "second stage" of aerobic, copper-catalyzed oxidation of ascorbic acid (6), and the formation of the methylene blue-reducing "diketogulonic acid" from dehydroascorbic acid which, according to Frankenthal (7) likewise depends upon the presence of phosphate.

Intramolecular Stabilization of Dehydroascorbic Acid

In the course of the present study it has become evident that at neutrality and anaerobically dehydroascorbic acid suffers two distinct spontaneous changes. The first consists in an intramolecular stabilization process which, according to Herbert *et al.* leads to diketogulonic acid (Equation 1). Ghosh and Rakshit (2) have proposed an alternative

$$(1, a) \quad \underset{\underset{H}{|}}{HOC} - \underset{\underset{H}{|}}{\overset{\overset{H}{|}}{C}} - \underset{\underset{H}{|}}{\overset{\overset{OH}{|}}{C}} - \underset{\underset{(OH)_2}{\wedge}}{C} - \underset{\underset{(OH)_2}{\wedge}}{C} \overset{\overline{\quad O \quad}}{=} O \rightleftharpoons HOC - \overset{\overset{H}{|}}{C} - \overset{\overset{OH}{|}}{C} = C \overset{\overline{\quad O \quad}}{\underset{O - H - O}{\parallel}} C = O$$

<center>(I)   (II)</center>
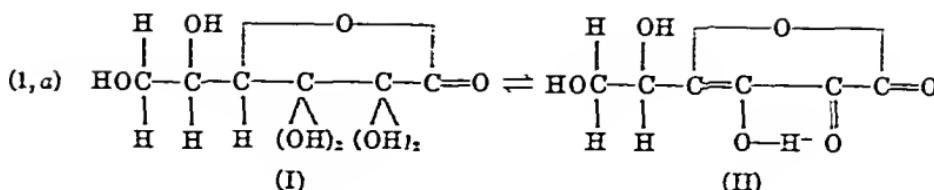
formulation of this stabilization process (Equation 1, a). The enol-lactone of diketogulonic acid replaces the open chain formula; the acidic properties are explained as arising from the reactivated hydroxyl at C_3. In the present work preference is given to the enol-lactone formula (II) as, among other observations, the requirement of 2 equivalents of base, one at pH 7.3 and a second, slowly, at pH 8.5 (opening of the lactone ring), is in better agreement with the new formula (II) than with diketogulonic acid. The latter, in the absence of oxalic acid formation, would require only 1 equivalent of base. In slightly alkaline solution the intramolecular stabilization of dehydroascorbic acid proceeds almost instantaneously; for this reason it is believed that the second spontaneous change originates from the intramolecular stabilization product (II) rather than from dehydroascorbic acid (I).

Irreversible Transformation

In alkaline solution dehydroascorbic acid or its stabilization product (II), respectively, suffers a different change. Our knowledge of this second spontaneous transformation is scanty and only qualitative. Herbert *et al.* in their classical paper state: "These alkaline solutions [N-alkali] are extraordinarily unstable... Some decomposition takes place, even in an inert atmosphere, with formation of oxalic acid." Judging from the degradation products, there is little doubt that this second, non-oxidative transformation constitutes an irreversible change.

In the present work evidence has been obtained that there exists a spontaneous and non-oxidative formation of oxalic acid from (II) at neutrality. In distinction to the spontaneous reaction in alkaline solution, this one requires for its occurrence the presence of phosphate which induces catalytically the rupture of the 6-carbon chain. It is the same reaction Borsook and coworkers have studied both *in vitro* and *in vivo* and recognized as an irreversible process.

If the molecular ratio of dehydroascorbic acid-phosphate is at least 1:5 and the initial dehydroascorbic acid concentration in the order of 1 mM per liter or less, conditions which are amply fulfilled in animal tissue, then the non-oxidative oxalic acid formation follows almost quantitatively Equation 2. The catalytic action of phosphate, which results in a shift of the non-catalyzed oxalic acid formation by about 4 pH units to the acid

$$(2) \quad HO-\overset{\displaystyle \overset{H}{|}}{\underset{\displaystyle \underset{H}{|}}{C}}-\overset{\displaystyle \overset{OH}{|}}{\underset{\displaystyle \underset{H}{|}}{C}}-\overset{\displaystyle \overset{}{|}}{\underset{\displaystyle \underset{O^-}{|}}{C}}=C-\overset{}{\underset{\displaystyle \underset{O}{\|}}{C}}-C=O \xrightarrow[\text{H}_2\text{O}]{\text{PO}_4 \text{ (pH 7)}} HOOC-COOH \ (+ \text{threose})$$

(II)
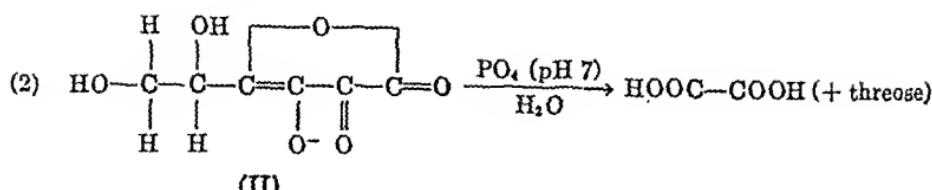
side, is not restricted to this anion; although systematic tests have not yet been carried out, it may be recorded that cyanide shows the same effect at much lower concentrations.

During the quantitative study of oxalic acid formation the interesting observation has been made that not all of the oxalic acid formed appears as "free" oxalic acid; part ($\geqq 10$ per cent) of it could be traced in the filtrate of the calcium oxalate (and phosphate) precipitate by treating the same for several hours at pH 8.5. As, in the absence of phosphate, no oxalic acid is formed at this pH from (II), this extra oxalic acid might be ascribed to an oxalyl precursor, possibly oxalyl threose. It appears, therefore, as if (II) suffers first hydrolytic scission of the carbon-carbon linkage at C_1 and C_2 and subsequently hydrolysis of the lactone ring. Much higher yields (40 to 80 per cent) of oxalyl compound were observed in corresponding aerobic experiments both with ascorbic and dehydroascorbic acids. In this case the oxalyl compound is oxalylthreonic acid which, according to the higher yield, seems to be less apt to hydrolyze than the corresponding oxalyl threose. The negative response of the furfural test given by the completely oxidized solutions proved the entire absence of any C_5 or C_6 compound, i.e. the absence of unchanged (II), and excluded the possibility of any other precursor to replace oxalylthreonic acid. Further evidence for the stability of the lactone ring of (II) is provided for by the existence of oxalyl compounds and, hence, further support is lent to the formula advocated above for the intramolecular stabilization product (II).

Dismutative Side Reaction

When Equation 2 for the mechanism of the irreversible transformation was presented above, an upper limitation (0.001 M) of its practical validity independent of the initial dehydroascorbic acid concentration was pointed out. This conclusion was drawn from experiments showing the influence of the initial dehydroascorbic acid concentration upon the course of the spontaneous transformation. At an initial concentration of 4.3 mM per liter a competitive side reaction was evident which was characterized by the formation of a reducing compound, the reducing power of which (against iodine) persisted in acid solution. Judged by its chemical and physical properties in solution, it might be ascorbic acid. In the absence of phosphate a similar observation has already been reported by Herbert et al.

The positive influence of the initial dehydroascorbic acid concentration upon the percentage of reducing compound produced indicated that this reaction is of a higher order. This justified the assumption of an interaction between 2 molecules of dehydroascorbic acid which results in the formation of a reducing compound, possibly ascorbic acid.

Further independent evidence in support of the dismutative nature of this side reaction was obtained from a study of the yellow-colored substance which appeared regularly at pH 7 in the course of the anaerobic, spontaneous transformation. It was perceived qualitatively that this yellow color became stronger the more reducing compound was formed and, hence, it was thought possible that it is derived from the oxidized partner of the reducing compound in the dismutative change. An oxidized derivative of (II) might feasibly display absorption in the visible region, particularly in neutral or slightly alkaline solution.

In order to elucidate the question of the existence of the presumed oxidized dismutation partner, the phenomenon of the yellow color was subjected to a quantitative colorimetric study. Its manifestations were expected to correspond quantitatively to those shown by the simultaneously formed reducing compound. This was indeed the case: the color intensity increased proportionally with the amount of reducing compound produced by the catalytic influence of increasing amounts of cyanide (see Fig. 3); increasing the initial dehydroascorbic acid concentration caused the color intensity to increase proportionally with the amount of reducing compound formed. The yellow substance was found to be unstable, and it faded considerably on standing at room temperature. This fact suggested a spontaneous disintegration which, in view of the accumulation of carbonyl groups in the oxidized dehydroascorbic acid molecule, is not surprising. In analogy to the products obtained by direct oxidation (1), oxalic acid and threonic acid are presumably arising out of this spontaneous disintegration. This presumed mechanism was strongly supported by the fact that more oxalic acid could be found than the irreversible transformation (Equation 2) could possibly yield. This instability made itself felt during the colorimetric measurements, which in some instances gave lower values than would have been expected if proportionality were fulfilled.

Treatment of the acidified equilibrium solutions with hydrogen sulfide gave rise to an additional reducing compound designated H_2S-recoverable reducing compound. Like the one obtained directly, it took up iodine in acid solution. It could be assumed that this compound originated from the yellow anion, since the amount of extra reducing compound approached the same value as has been obtained by direct iodometric titration (*i.e.* the reducing compound formed by dismutation). This fact is in agreement with the expectation that the oxidized partner of a dismutative reaction (in

this case measured iodometrically after reduction with H_2S) must have the same concentration as its reduced counterpart. In the majority of cases, however, the amount of H_2S reducing compound was found markedly less than is required by the above theory. Although the deficit of this reducing compound could be explained by assuming that part of the yellow compound has suffered decomposition and has thus escaped reduction by H_2S, such an explanation is not entirely satisfactory. Further independent data were required which would demonstrate that the deficit of H_2S-recoverable reducing compound is balanced by an equivalent amount of oxalic acid originating, as already mentioned, from the spontaneous disintegration of the yellow compound. Such data have in fact been obtained from experiments in which, besides reducing compound and H_2S-recoverable reducing compound, the total oxalic acid was determined. These data (Table II) show clearly that the deficit of the H_2S-recoverable reducing compound is of the same order as an amount of oxalic acid which has been found in excess to that expected from the irreversible transformation alone.

The scheme shown in Equation 3 is given as an illustration of the dis-

mutative side reaction. The formulation of the yellow anion as triketo-gulonic acid lactone is, of course, only tentative; it should express the intense absorption band at $\lambda_{max.}$ 410 mμ and its reducibility by hydrogen sulfide into a reducing compound, possibly ascorbic acid.
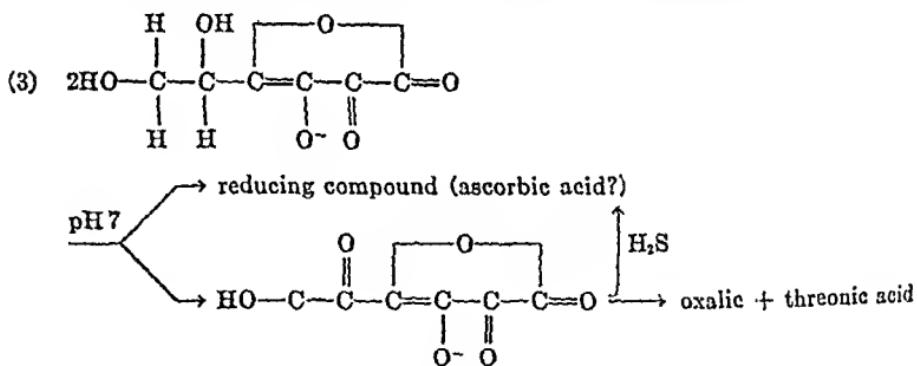
<div align="center">EXPERIMENTAL</div>

<div align="center">*Methods and General Procedure*</div>

While we followed in principle the Thunberg technique of Borsook and coworkers, it seemed advisable, in order to establish a reliable quantitative relationship between dehydroascorbic acid and its spontaneous transformation product or products, to use larger quantities of dehydroascorbic acid. Particularly with regard to the formation of a compound the reducing power of which persisted in acid solution, as found by Herbert *et al.* (1), this

measure seemed to be essential. The applicability of the Thunberg technique was, however, restricted to well buffered reaction mixtures and to relatively slow reactions. The necessity of investigating the transformation at pH 7.4 in the absence of phosphate buffer, and the desirability of giving a quantitative account of the color formed during the reaction, demanded a method of wider applicability. This was found in the use of cyanide, thiocyanate, and 8-hydroxyquinoline as auxiliary compounds; these substances, as has been demonstrated by Barron and coworkers (8) and others, inhibit practically completely the catalytic effect of copper traces upon the autoxidation of ascorbic and dehydroascorbic acids.[1] Yet only thiocyanate and 8-hydroxyquinoline proved to be without influence upon the course of the spontaneous transformation such as proceeds in a vacuum. Cyanide behaved differently; its influence is treated in a separate section.

Oxalic Acid Estimation—During the separation of free oxalic acid as the calcium salt the use of strongly ammoniacal solutions was avoided in order to exclude non-catalyzed oxalic acid formation from unchanged (stabilized) dehydroascorbic acid. Therefore, at the end of the spontaneous reaction, the solution (or an aliquot part) containing one of the above auxiliary substances, *e.g.* 8-hydroxyquinoline, was adjusted to pH 5 with an appropriate amount of acetic acid. Excessive calcium chloride solution was added to precipitate both oxalate and phosphate and the precipitate was allowed to stand overnight in the refrigerator. Apart from making the influence of atmospheric oxygen negligible, the pH chosen caused the coprecipitated calcium phosphate to assume crystalline form and thus permitted the easy removal of soluble substances which interfere with the subsequent permanganate titration. After the solution had stood in the cold, the precipitate was decanted through a quantitative filter and the filtrate kept for the recovery of oxalyl compounds. The precipitate was then washed twice with calcium oxalate water and once with distilled water, the washings being discarded. Decomposition of the oxalate with hot 6 N sulfuric acid and titration with standard permanganate were carried out according to known procedures.

Test Example—A solution containing 3.57 mg. of oxalic acid and 7 mg. of ascorbic acid, phosphate buffer, and 1 mg. of NaCN was treated according to the above procedure. The decomposed oxalate required 0.520 (0.500) ml. of a standard permanganate solution; 0.504 ml. of the same solution was used for the direct titration of 3.58 mg. of oxalic acid.

Oxalic Acid from Oxalyl Compounds—The filtrate from the first calcium oxalate (and phosphate) precipitate, exclusive of the washings, was made just alkaline towards phenolphthalein with ammonia (pH 8.5) and kept for

[1] Unpublished experiments.

4 hours at room temperature (25°) in a current of oxygen-free nitrogen. The faint pink color was occasionally restored with a drop of dilute ammonia. Subsequently the pH was readjusted to 5 with acetic acid, and after addition of some more calcium chloride solution the precipitate was allowed to stand overnight in the refrigerator. The above procedure of separation and titration of the calcium oxalate was then followed. Repetition of the hydrolysis at pH 8.5 with the second filtrate gave, as a rule, only an insignificant amount of oxalic acid and has, therefore, not been carried out in routine work.

Estimation of Reducing Compound—After the spontaneous change had gone to completion, the solution or an aliquot part thereof was made acid (pH 1) with 6 N sulfuric acid and then titrated with 0.01 N iodine in the usual manner. Assuming an uptake of 2 atoms of iodine per molecule of reducing compound formed, if not stated otherwise, the result was expressed as reducing compound in per cent of that amount of ascorbic acid which originally had been submitted to oxidation with iodine.

Preliminary Experiments

Acidity of Dehydroascorbic Acid—For the study of the spontaneous changes at pH 7.4 the acid (hydrogen iodide) dehydroascorbic acid solutions had to be adjusted to this pH with an appropriate neutralizing and, if desired, buffering mixture. The composition of such a mixture would differ according to whether dehydroascorbic acid is a neutral substance (Herbert *et al.* (1)) or has the same acidity as ascorbic acid (Ghosh and Rakshit (2)). The English authors did not carry out a pH control, whereas Ghosh and Rakshit did, but their first record of acidity was given only 20 hours after the beginning of the platinum-catalyzed oxidation of ascorbic acid. Thus it became clear that both views had to be tested by an independent procedure. By the method of Moll and Wieters (9), pure dehydroascorbic acid (*i.e.* free of electrolytes) could be prepared more rapidly with quinone as oxidizing agent; hydroquinone and excess quinone were subsequently removed by ether extraction. 45 minutes after the oxidation with quinone, etc., the pH of the dehydroascorbic acid solution was 3.33 as compared with 2.92, the pH of an ascorbic acid solution of the same molar strength (0.068 M). However, in distinction to the pH of the ascorbic acid solution that of dehydroascorbic acid did not remain constant; the acidity increased relatively rapidly during the first 24 hours and then more slowly, as is demonstrated in Fig 1. For comparison the pH values published by Ghosh and Rakshit for M/120 dehydroascorbic acid have been plotted on the same graph. Extrapolation of the experimental values to zero hour would undoubtedly yield the pH of a neutral substance, in confirmation of Herbert, Hirst, *et al.*, particularly if one takes into considera-

tion that the dehydroascorbic acid under test still contained 1.2 per cent unchanged ascorbic acid. Indeed, repetition of the corresponding experiment of Herbert *et al.* with electrometric control immediately after exact neutralization of the hydrogen iodide gave pH 6.44. The discrepancy between results obtained by Herbert *et al.* and Ghosh and Rakshit is only an apparent one; the latter authors have measured the acidity of dehydroascorbic acid at a time when it had already assumed acidic properties owing to the spontaneous intramolecular stabilization process. The results show, furthermore, a definite decrease of pH between 20 and 72 hours of observation; *i.e.*, the increase of acidity reached (again in agreement with
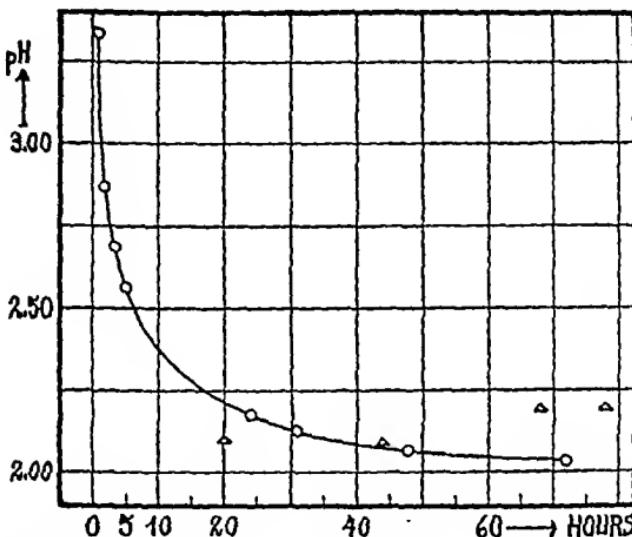
Fig. 1. Acidity of freshly prepared dehydroascorbic acid (○); results published by Ghosh and Rakshit (△).

Herbert *et al.*) constancy simultaneously with the change of specific rotation.

Thiocyanate and 8-Hydroxyquinoline As Auxiliary Compounds during Spontaneous Transformation—The introduction of auxiliary compounds into the study of chemical reactions, particularly into those of biological interest, has a serious drawback, as it may be objected that they render the reaction atypical. Therefore, proof has to be given that the course of the reaction under study is not altered in the presence of such compounds. It is the object of this experimental series to demonstrate that both thiocyanate and 8-hydroxyquinoline have no influence other than that expected from their copper-binding ability.

To this end increasing amounts of the above copper complex formers were added to a constant mixture of dehydroascorbic acid and phosphate

buffer with a final pH of 7.4 and a constant total volume. The amount of reducing compound formed at the end of the spontaneous change was expected to be independent both of the type and of the concentration of the auxiliary reagent employed. In an analogous experiment carried out with the Thunberg technique the yield of reducing compound should be of the same order as in the aerobic experiments. The results will show that this is the case.

In connection with the adjustment of the reaction mixture to pH 7.4 it was assumed, in accordance with the result from the preceding experiment, that neutralization of the HI formed during the oxidation of ascorbic acid and addition of sufficient buffer of pH 7.4 would give the desired initial acidity. This was, however, not the case; owing to the intramolecular stabilization and formation of the acidic enol-lactone of diketogulonic acid, the initial pH was slightly more acid. The correct initial pH 7.4 was only obtained after the addition of an amount of secondary phosphate which by itself was sufficient to adjust the corresponding amount of ascorbic acid to pH 7.4. Were it not for the decisive result obtained from the preceding experiment, one would believe that freshly prepared dehydroascorbic acid has the same acidity as ascorbic acid. The truth, however, is that at pH 7.9 the change into the acidic enol-lactone is almost instantaneous.

Thiocyanate Procedure—In each of a series of 25 ml. Erlenmeyer flasks the following neutralization and buffering mixture was placed: 1.58 ml. of 0.1 N NaOH (for the neutralization of the HI formed during the oxidation of 14 mg. of ascorbic acid), 1.26 ml. of 0.2 M secondary phosphate (adjustment of 14 mg. of ascorbic acid to pH 7.4), an additional 5.0 ml. of 0.2 M secondary phosphate buffer, pH 7.4. The final phosphate molarity was 0.068.

The following amounts of 0.1 M potassium thiocyanate were added to each Erlenmeyer flask respectively: 0.1, 0.2, 0.4, 0.6, 0.8, 1.6, 3.2, 6.4 ml. Distilled water was added to make the volume 16.0 ml. To each set were added 2.50 ml. of dehydroascorbic acid solution containing 14.0 mg. of ascorbic acid oxidized by iodine. The mixed solutions were allowed to stand for 5 hours at room temperature (28°).

The amount of reducing compound observed was 12.50 ± 0.02 per cent in all eight different thiocyanate concentrations under test. The pH of the control immediately (25 seconds) after mixing was pH 7.43; it dropped to pH 7.25 after 4 hours and then remained practically constant.

8-Hydroxyquinoline—The procedure was the same as for thiocyanate except that 1.5, 3.0, and 6.0 mg. of 8-hydroxyquinoline were used and the flasks were incubated for 3 hours at 37°. The yield of reducing compound amounted to 11.35 ± 0.01 per cent.

Thunberg Series—According to the technique described by Borsook *et al.*

14.0 mg. of ascorbic acid (3.95 ml.) were neutralized in a Thunberg tube with 3.50 ml. of phosphate buffer, pH 7.9. A series of six such tubes was incubated for 24 hours at 37°. Four tubes showing a gold-yellow color, which faded completely on acidification, gave an average yield of 8.6 ± 0.9 per cent of reducing compound. The remaining tubes were brownish yellow (air leakage?) and gave lower yields (3.3 and 1.7 per cent) of reducing compound. The lower values (8.6 per cent), compared with 11 to 12 per cent above, are explained by both the lower final pH (6.5) and the ratio of phosphate to dehydroascorbic acid of 8:1, as compared with 16:1, apart from the improved exclusion of oxygen by the new method.

Experiments Referring to Equations 1, a and 2

Spontaneous Transformation of Dehydroascorbic Acid at pH 7, in Absence of Phosphate—In a tall 100 ml. beaker 176.2 mg. of ascorbic acid were oxidized with 20.0 ml. of 0.1 N iodine solution. The influence of oxygen at neutrality was excluded by the addition of 20 mg. of 8-hydroxyquinoline in 0.1 ml. of lukewarm ethanol to the colorless dehydroascorbic acid solution. Addition of 20.1 ml. of 0.1 N sodium hydroxide for the neutralization of the hydrogen iodide formed during the oxidation was taken as zero hour of the beginning of the spontaneous transformation. The first pH reading 25 seconds after the start of the reaction corresponded to pH 6.44 at 27°. 0.1 N NaOH was then added to pH 7.28, which was closely maintained by the addition of further amounts of NaOH. At 18 minutes 8.0 ml. of 0.1 N NaOH were used up, equivalent to 80 per cent of a new acidic group. The reaction became now progressively slower; after 3 hours, there was no further increase of acidity. The total increase of acidity was equivalent to the formation of a new hydrion from 1×10^{-3} mole of dehydroascorbic acid. Before analysis of the equilibrium solution, it was transferred into a 100 ml. measuring flask.

When the procedure given at the beginning of the experimental section was employed, no "free" oxalic acid was found to be present. Likewise, adjustment to pH 8.5 of 25 ml. of the equilibrium solution did not reveal the presence of any oxalyl compound, even after 48 hours standing.

It was, however, observed that while standing (in the cold) the solution had turned acid by more than 1 pH unit and the maintenance of pH 8.5 required the gradual addition of alkali. After the total addition of 2.40 ml. of 0.1 N NaOH the pH remained constant. This addition of further alkali corresponded to the neutralization of a 2nd hydrion which, as no precipitate of calcium oxalate appeared on further standing, belonged to diketogulonic acid formed from the corresponding lactone. The presence of 2.4 per cent of reducing compound was observed.

H₂S-Recoverable Reducing Compound—In an Erlenmeyer flask 20 ml. of

solution were acidified with 1 ml. of 6 N sulfuric acid and treated for 20 hours with hydrogen sulfide under slight pressure. H_2S was expelled from the solution by a current of oxygen-free nitrogen; after 6 hours the lead acetate test for H_2S was negative. Iodometric titration revealed the presence of 35.3 per cent of reducing compound which includes 2.4 per cent of reducing compound formed by dismutation.

Spontaneous and Irreversible Transformation at pH 7, in Presence of Phosphate, Anaerobically—In the upper part (approximately 50 ml. capacity) of a specially constructed large Thunberg vessel 0.8986 gm. (= 0.511 $\times 10^{-2}$ mole) of dry ascorbic acid was placed and oxidized with 9.90 ml. of a 1.026 N iodine solution. The resulting acid solution still showed the color of free iodine.[2] The lower part of the Thunberg vessel, having a total capacity of 250 ml., contained 4.75 gm. of $Na_2HPO_4 \cdot 2H_2O$, 0.27 gm. of KH_2PO_4, and 7.30 ml. of 1.39 N NaOH in a total volume of 100 ml. After thorough evacuation with an oil pump the contents of the upper and lower part were mixed. A colorless solution resulted which, after a short time, turned yellow and then orange. The reaction was allowed to proceed for 20 hours at 37°, after which the vacuum was released with CO_2. In order to make the solution inert towards oxygen 5 ml. of 0.1 N KCNS were added and the solution then transferred to a 200 ml. measuring flask.

Result—Free oxalic acid, 0.311 $\times 10^{-2}$ mole from 0.511 $\times 10^{-2}$ mole of dehydroascorbic acid; oxalic acid from oxalyl compound, 0.035 $\times 10^{-2}$ mole; reducing compound, 0.116 $\times 10^{-2}$ mole (22.7 per cent); H_2S-recoverable reducing compound, 0.183 $\times 10^{-2}$ mole which includes the above 0.116 $\times 10^{-2}$ mole of reducing compound.

In connection with the possible identity of the reducing compound with ascorbic acid a furfural distillation was carried out with 30 ml. of the equilibrium solution. The furfuraldehyde was estimated as the brick-red 2,4-dinitrophenylhydrazone (10); 11.6 (10.8) mg. of hydrazone were obtained, m.p. 183°. 23.1 mg. of ascorbic acid treated in the same manner gave 22.8 mg. of hydrazone, m.p. 197–199°. The mixed melting point observed was 189°, indicating that the crude hydrazone obtained from the test solution presented, though impure, principally the same compound as could be obtained from authentic ascorbic acid. The yield of ascorbic acid, as calculated from the yield of furfural-2,4-dinitrophenylhydrazone, is lower than expected from the iodometric titration value in acid solution; the identity of the reducing compound with ascorbic acid must, therefore, be considered with due reserve until further evidence, *e.g.* quantitative spectroscopic analysis, is available.

[2] This observation, which indicates the existence of a true chemical equilibrium, corresponds to the observation made by Herbert, Hirst, *et al.* (1) about the formation of free iodine when dehydroascorbic acid solutions were evaporated in the presence of an equivalent amount of hydrogen iodide.

Irreversible Transformation Catalyzed by Cyanide, at pH 7, in Absence of Phosphate—Preliminary tests with increasing amounts of cyanide have shown that both the rate of formation and the yield of reducing compound increased far beyond the values observed with thiocyanate or 8-hydroxyquinoline. As those experiments have been carried out in the presence of phosphate, it could not be said with certainty whether the action of cyanide depends upon the presence of phosphate, or whether cyanide exerts independently an analogous influence upon the spontaneous transformation, as is shown by phosphate.

In a tall 100 ml. beaker 177.2 mg. of ascorbic acid (1.01×10^{-3} mole) were oxidized with the calculated amount of 0.1 N iodine; then 2.0 ml. of NaCN solution (0.197 M) were added. The acid mixture was finally neutralized with 16.16 ml. of 0.1 N NaOH, which takes into account the neutralizing power of the NaCN used.

The pH of the solution was measured immediately after neutralization. Owing to the fast reaction the initial reading is only approximate; *i.e.*, pH 3.7 ± 0.2. 5 minutes after the addition of 7.20 ml. of 0.1 N NaOH was begun, the pH was 7.1. The solution assumed now an intense yellow color, and the acidity still increased, though more slowly. The reaction was considered to be practically finished after 180 minutes, when the increase of acidity during the past hour amounted only to 0.20 ml. of 0.1 N NaOH. The total amount of NaOH which had been used in addition to the neutralization of hydrogen iodide was found to be 14.1 ml., which is 41 per cent more than was obtained under the same experimental conditions without cyanide. This observation indicated that free oxalic acid had already formed. Free oxalic acid, 0.21×10^{-3} mole; oxalic acid from oxalyl compound, 0.09×10^{-3} mole. Thus 0.30×10^{-3} mole of oxalic acid has been formed from 1.01×10^{-3} mole of dehydroascorbic acid. Iodometric titration in acid solution gave 28.6 per cent (0.29×10^{-3} mole) of reducing compound. An identical experiment with cyanide (0.004 M) which had been carried out in the presence of 0.1 M phosphate gave 27.9 per cent of reducing compound. A higher value would have been expected if the catalytic effect of phosphate is added to that exerted by cyanide. The result obtained suggests a stronger affinity of cyanide towards dehydroascorbic acid, for in the presence of both phosphate is displaced by cyanide.

If it is assumed that the reducing compound has been formed by a dismutative change, 0.43×10^{-3} mole of total oxalic acid would have been expected by difference.

Formation of Oxalyl Compounds during Aerobic, Copper-Catalyzed Oxidation of Ascorbic and Dehydroascorbic Acid. Oxidation of Ascorbic Acid with Oxygen—1.7294 gm. of ascorbic acid (= 0.982×10^{-2} mole) were placed in a dry absorption vessel (the type used for catalytic hydrogenations), the air was replaced by oxygen, and an aqueous solution of 8.49 gm. of Na₂-

$HPO_4 \cdot 2H_2O$, 0.57 gm. of KH_2PO_4, and 14.8 mg. of $CuCl_2 \cdot 2H_2O$ was added, making the total volume 113.5 ml. The vessel was shaken at room temperature (30.5°; 754 mm. of Hg). After 4 hours the oxygen uptake had stopped. In agreement with the observations made by Heard and Welch (11) the oxygen uptake amounted to 1.345×10^{-2} mole; for a 3 atomic oxidation 1.472×10^{-2} mole was calculated.

Aerobic Oxidation of Dehydroascorbic Acid—Into the main part of each of three Warburg vessels containing the requisite amounts of neutralizing phosphate mixture and a trace of copper chloride, 2.50 ml. of dehhydro-ascorbic acid solution were allowed to flow from the side cups after temperature equilibrium had been reached. Final phosphate molarity 0.13 M, pH 7.4; amount of dehydroascorbic acid 3.98×10^{-5} mole; total volume 8.0 ml.; temperature 25°.

The oxygen uptake was relatively slow and corresponded to the low concentration of free copper ions in the presence of excess iodide. After 27 hours the oxygen uptake had ceased, amounting to 2.89, 2.92, and 2.92×10^{-5} mole; *i.e.*, a complete oxidation to the threonic acid stage and about 50 per cent oxidation towards a second oxidized derivative of threonic acid.

The results of both experiments as far as oxalic acid and oxalyl compound formation are concerned are as follows: (*a*) 0.982×10^{-2} mole of ascorbic acid produced 0.391×10^{-2} mole of free oxalic acid and 0.339×10^{-2} mole as oxalyl compound; over-all yield 74.4 per cent; (*b*) 3.98×10^{-5} mole of dehydroascorbic acid produced 0.73×10^{-5} mole of free oxalic acid and 3.19×10^{-5} mole as oxalyl compound; over-all yield 98.5 per cent.

It may be added that following the furfural test with the oxidized ascorbic acid solution (*a*) a negative response was observed which proved the absence of any transformation product having an intact 6-carbon chain. Control distillation with 171.6 (198.8) mg. of iodine-oxidized ascorbic acid gave a brown 2,4-dinitrophenylhydrazone, m.p. 95° (unsharp), weighing 82.0 (103.0) mg., thus showing that distillation in the presence of 12 per cent HCl may also be of diagnostic value with dehydroascorbic acid.

Experiments Concerning Dismutative Side Reaction (Equation 3)

Qualitative Spectroscopic Identification of Reducing Compound—The reducing compound which arises spontaneously out of dehydroascorbic acid and which reduces iodine in acid solution has most likely a dienol group, like ascorbic acid. Titration of dichlorophenol indophenol with solutions containing the reducing compound (at pH 2, in the presence of HPO_3) gave values which were comparable with those obtained iodometrically, though they were generally lower. Yet even this additional observation did not permit us to conclude whether the reducing compound is identical

with ascorbic acid. The chemical tests in solution lack specificity in this respect. A qualitative spectroscopic test in the ultraviolet region showed, however, the presence of a strong absorption band at 268 to 272 mμ, which at pH 7.4 is characteristic for ultraviolet absorption of ascorbic acid. This result has been obtained from a 0.8×10^{-4} M solution of dehydroascorbic acid, in the presence of phosphate and 5 mg. of NaCN per 50 ml. of solution. It can be assumed that at least part of the reducing compound formed is ascorbic acid.

Influence of Initial Dehydroascorbic Acid Concentration upon Formation of Reducing Compound—The concentrations of dehydroascorbic acid used in this work varied between 4.30 and 90 mM per liter, which, for reasons mentioned above, were higher than those employed by Borsook and co-workers (0.6 to 1.7 mM per liter) and far above the concentrations of dehydroascorbic acid present in animal tissues. As it was ultimately desired to obtain information about the chemical mechanism by which vitamin C is destroyed in the living organism, the knowledge of the influence of the initial concentration was imperative to permit valid conclusions to be drawn in the biological range of dehydroascorbic acid concentrations. If the reducing compound formed during the spontaneous transformation is a dismutation product, *i.e.* a product formed by the interaction of 2 molecules of dehydroascorbic acid, a positive influence of the change in concentration is anticipated.

The Tunberg technique described above has been applied to concentrations $>5 \times 10^{-3}$ M. The value corresponding to 4.3×10^{-3} M has been obtained from the experiments in the presence of 8-hydroxyquinoline. The amount of reducing compound found by iodometric titration in acid solution was plotted in per cent of the ascorbic acid originally submitted to oxidation against the initial dehydroascorbic acid concentration in mM per liter (Fig. 2). The positive increase of the relative formation of the reducing compound with increasing initial dehydroascorbic acid concentration is in agreement with the assumption that the dismutation of dehydroascorbic acid is a reaction of higher order; therefore, at infinitely low initial concentration, no ascorbic acid is formed by dismutation.

Interdependence of Reducing Compound and Yellow Compound Formation—The catalytic effect of cyanide upon the dismutative transformation of dehydroascorbic acid offered a welcome opportunity to decide experimentally whether the yellow color often observed belongs to the hypothetical oxidized counterpart of the reducing compound, or whether it is merely a by-product without significance. The first alternative would be the more correct one if, by alteration of the external experimental conditions, an increase or decrease of the amount of reducing compound is

followed by a proportional change of the color intensity. The following experiments were designed to prove the expected interdependence of the dismutation partners.

The experimental procedure is in principle identical with that described in the presence of thiocyanate. A parallel series of 25 ml. Erlenmeyer flasks contained 14 mg. of oxidized ascorbic acid (final molarity 0.43×10^{-2}), 1.26 ml. of 0.2 M secondary phosphate, and 9.0 ml. of 0.2 M phosphate buffer, pH 7.4 (final phosphate molarity 11.1×10^{-2}). Different amounts of 0.22 M NaCN solution were further added, giving a final NaCN concentration which varied between 5.95×10^{-4} and 1.07×10^{-1} M. Neutraliza-
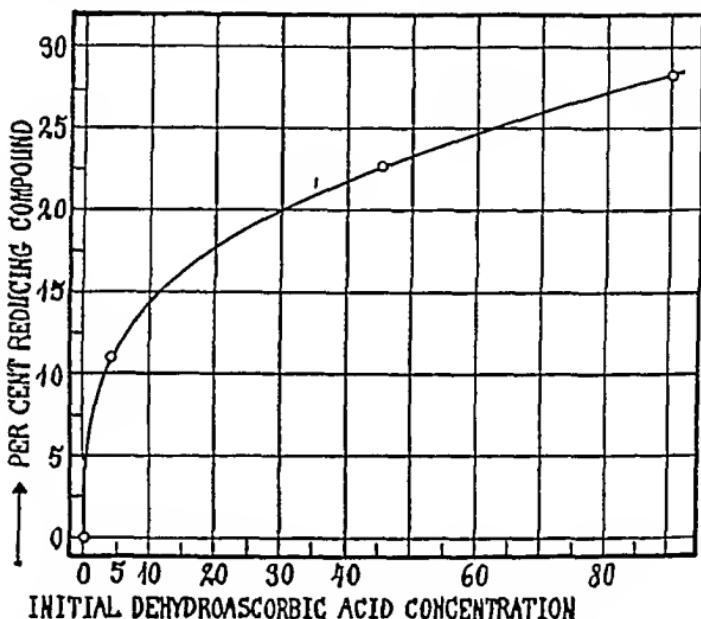
FIG. 2. Influence of the initial dehydroascorbic acid concentration (mM per liter) upon the formation of the reducing compound.

tion of the HI was adjusted either by the addition of 0.1 N NaOH or HCl, according to whether the amount of cyanide used was insufficient or in excess. The total volume was in all cases 18.50 ml. The temperature was 25°. At the end of the transformation the color was measured with a Pulfrich step-photometer having a cuvette of 30 mm. depth and a No. 43 filter (effective optical density at center 434 mμ). The corresponding duplicates were acidified after 3 hours standing and the reducing compound estimated iodometrically. The results obtained were plotted in Fig. 3 (Curve B) as mM per liter of reducing compound formed by dismutation, and (Curve A) as color intensity, $\log I_0/I$ against the corresponding cyanide molarity.

The concentration of the oxidized dismutation product as measured by the increase of its yellow color at pH 7.4 increases proportionally with the cyanide concentration up to 2.4×10^{-3} M. Up to the same cyanide concentration the amount of reducing compound formed likewise increases proportionally. At higher cyanide concentrations no further increase of either the color intensity or the reducing compound is observed. The maximum values remain fairly constant up to 3.6×10^{-3} M NaCN in the case of the yellow compound, and more precisely constant up to 5.0×10^{-3} M CN in the case of the reducing compound. At this concentration the yellow color has faded to 59 per cent of its original maximum strength and continues to fade with further increase of the cyanide concentration.
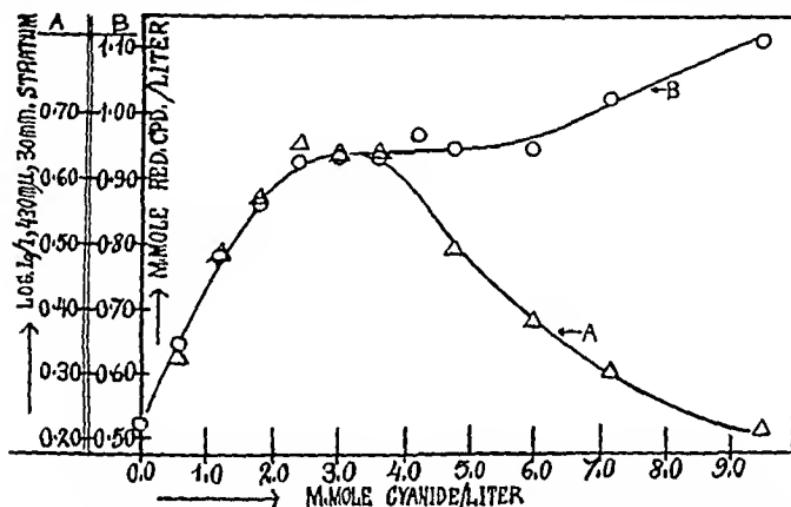
FIG. 3. Influence of increasing cyanide concentration upon the formation of the reducing compound (Curve B) and upon the color intensity of the yellow, oxidized dehydroascorbic acid (Curve A).

The amount of reducing compound, on the other hand, again showed an increase which ceased at 0.071 M CN, with a total yield of 60 per cent of reducing compound. A yield of 50 per cent would have corresponded to a complete dismutative change of dehydroascorbic acid. No explanation can be given at present for the higher value observed. The decrease of the color intensity suggests that at concentrations $\geqq 3.6 \times 10^{-3}$ M cyanide acts upon the colored dismutation product. The upper limit of the catalytic action of cyanide seems to be 2×10^{-3} M.

Increase of Relative Color Intensity with Increasing Initial Dehydroascorbic Acid Concentration—As in the case of the reducing compound above, the same influence of the initial dehydroascorbic acid concentration is expected to take place with its yellow counterpart. To obtain evidence for this

assumption a series of experiments with increasing amount of dehydro-ascorbic acid for a given constant volume has been carried out.

To three pairs of 25 ml. Erlenmeyer flasks, each containing 7.0, 14.0, and 28.0 mg. of iodine-oxidized ascorbic acid and the requisite amount of neutralizing and buffering mixture to obtain pH 7.4, 0.35 ml. of 0.2 M NaCN and water are added to make a final volume of 18.5 ml.; the transformation was allowed to go to completion at 25°. The quantitative colorimetric estimation of the yellow color and the titration of the reducing compound were carried out as usual. The results are given in Table I.

Optical Absorption Characteristics of Yellow-Colored Anion—Only casual reference has been made in the literature to the yellow compound, its hue being given as brownish yellow (3). Since it is a new chemical entity, its properties are described in detail. As regards its optical characteristics the following observations have been made at pH 7.4 with a solution containing in 23.3 ml. 28 mg. of oxidized ascorbic acid, 0.4 ml. of 0.22 M NaCN, and

TABLE I

Influence of Increasing Initial Dehydroascorbic Acid Concentration upon Dismutative Formation of Reducing and Yellow Compounds

Dehydroascorbic acid, M per l.................	2.15×10^{-2}	4.30×10^{-2}	8.60×10^{-2}
Reducing compound, M per l.......	0.42×10^{-2}	1.00×10^{-2}	2.22×10^{-2}
Color intensity as log I_0/I, 10 mm. stratum, Filter 430..............	0.058	0.243	0.699
Log I_0/I per mole dehydroascorbic acid per liter....................	27.0	56.5	81.5

6.65 ml. of 0.2 M phosphate buffer. The solution was observed in a Pulfrich step-photometer at 26°; depth of solution 30.3 mm. As can be seen from the following figures the transparency increases rapidly with increasing wave-length.

Absorption, $m\mu$.... | 430 | 470 | 500 | 530 | 570 | 610 | 660 | 720 | 750

Log I_0/I.......... 2.100 0.409 0.045 0.010 0.008 0.010 0.020 0.026 0.033

A qualitative spectroscopic test showed the presence of a narrow and strong absorption band between 393 and 430 mμ, with the maximum at 409 to 410 mμ. The yellow color is unstable and fades on standing for 18 hours at room temperature to approximately half its original value. At dilutions corresponding to log I_0/I values between 0.925 and 0.072, with Filter 43, the yellow color follows the law of Beer, a fact which permitted the evaluation of the colorimetric measurements in terms of concentration values.

H$_2$S-Recoverable Reducing Compound—H$_2$S treatment of the equilibrium

solutions gave rise to an additional reducing compound which showed the same reducing properties towards iodine as the one formed by dismutation. The precursor of this additional reducing compound may be either the intramolecular stabilization product of dehydroascorbic acid, *viz.* the enol-lactone of diketogulonic acid, or the oxidized counterpart of the dismutative reducing compound, *viz.* the yellow enol-lactone of triketogulonic acid, according to whether the transformation follows the reversible (Equation 1, *a*) or the irreversible change (Equations 2 and 3).[3] The existence of the first precursor, the (stabilized) dehydroascorbic acid, is well founded (1), whereas the claim of the yellow anion as being another possible precursor of the H₂S-recoverable reducing compound is, *a priori*, purely hypothetical and needs further discussion.

Owing to the instability of the yellow anion this hypothesis cannot be subjected to a direct experimental test.   Indirect evidence, however, could be obtained through evaluation of the analytical data of the irreversible transformation.   In the theoretical considerations only one supposition has been made; *viz.*, the amount of reducing compound appearing at the end of the transformation has been formed by a dismutative reaction and accounts therefore for 2 molecules of dehydroascorbic acid in the balance. In preceding experiments presented in this section the validity of this supposition has been tested and found to be correct.   Hence it is expected that the amount of H₂S-recoverable reducing compound must be equal to the amount of reducing compound formed by dismutation.   It is further expected that, in consideration of the instability of the yellow anion, the extra amount of H₂S-recoverable reducing compound may be less, but must never surpass the amount of direct titratable reducing compound, provided, of course, that the transformation is rendered completely irreversible through the presence of sufficient phosphate (or cyanide).

All experimental observations made in this respect complied with these expectations.   For example Fig. 4 shows the result of a series of experiments which were originally designed to demonstrate the influence of increasing amounts of cyanide upon the dismutative formation of the reducing compound; the influence upon the H₂S-recoverable reducing compound was measured simultaneously.   The experimental procedure is exactly the same as that outlined above, which led to Fig. 3, the only difference being a lower phosphate molarity; *viz.*, 2.44×10^{-2} M.

At NaCN concentrations up to 2×10^{-3} M the observed deviation from the theoretically expected H₂S-recoverable reducing compound is probably due to the instability of the yellow compound from which it is supposed to derive, whereas the striking drop of the curve at NaCN concentrations $>2 \times 10^{-3}$ M, in close resemblance to the observations made with the

[3] Needless to say, both changes may occur side by side.

colorimetric tests (Fig. 3, Curve A), may be ascribed to the chemical inter-
action of CN′ which probably resulted in the formation of a non-reducible
(colorless) addition product.

In Table II typical examples of H₂S-recoverable and directly titratable
reducing compound are listed, as well as analytical data of the correspond-
ing experiments. Table II is particularly instructive, since it demonstrates
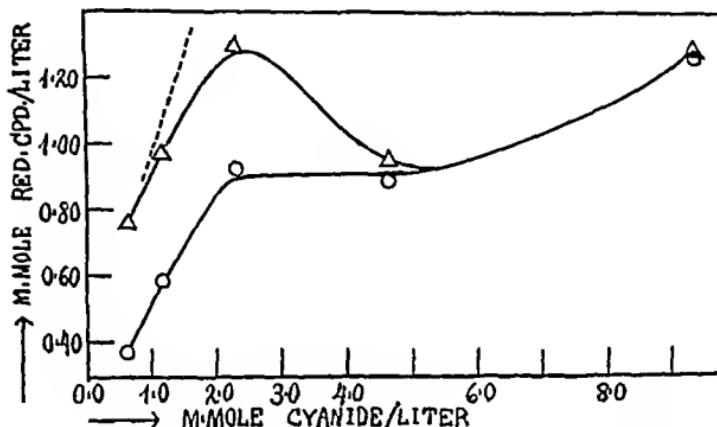
FIG. 4. Influence of increasing cyanide concentration upon the formation of both
the reducing compound (O) and the amount of extra reducing compound, obtained
after treatment with H₂S (△). The dotted line indicates the theoretically expected
(maximum) amount of H₂S-recoverable reducing compound.

TABLE II

*Mole of Transformation Products Obtained per Mole of Dehydroascorbic Acid,
Anaerobically, at pH 7 and in Presence of Phosphate*

Initial dehydroascorbic acid concentration	Reducing compound by dismutation	Reducing compound after H₂S treatment	Irreversible change calculated, $1 - 2 \times (b)$	Oxalic acid formed, $1 - 2 \times (b)$	Total oxalic acid		Excess oxalic acid, $(f) - (e)$	Deficit of H₂S-recoverable reducing compound, $2(b) - c$
					Found	Calculated, $1 - (c)$		
(a)	(b)	(c)	(d)	(e)	(f)	(g)	(h)	(i)
0.05	0.227	0.358	0.546	0.546	0.676	0.642	0.130	0.096
0.09	0.282	0.374	0.436	0.436	0.635	0.626	0.199	0.190

the normal deficit of the H₂S-recoverable reducing compound. As the excess
of oxalic acid found (Column h) is of the same order as the corresponding
deficit of H₂S-recoverable reducing compound (Column i), it can be assumed
that the oxidized partner of the dismutative change, being unstable, has
suffered partial decomposition, with the formation of oxalic and possibly
threonic acid, whereas the intact molecule gave rise only to the reducing
compound upon H₂S treatment.

DISCUSSION

The chemical facts about the irreversible transformation reported in this work need no further comment, since the quantitative formation of oxalic acid in the presence of phosphate is self-explanatory, at least as far as the irreversibility of the reaction is concerned. Whether or not this process involves the simultaneous formation of threose is a matter of secondary importance, which still awaits experimental confirmation.

The peculiar action of phosphate, on the other hand, which causes the spontaneous scission of a carbon-carbon linkage is less easily understood. In analogy to existing chemical facts it may perhaps be assumed that phosphate ions become temporarily associated with C_2 or C_3 of the dehydro-ascorbic acid molecule and thus, by increasing the negative charge there, cause the spontaneous rupture of the C—C linkage. The fact that the reactive cyanide ion exerts the same influence as phosphate and, furthermore, showed signs of possible chemical interaction with dehydroascorbic acid, can be quoted in favor of this explanation.

It has become clear now that the spontaneous increase of acidity during the irreversible transformation must be ascribed mainly to oxalic acid and, to a small extent, to oxalyl compounds but not to diketogulonic acid. Even in the case of the spontaneous intramolecular stabilization of dehydroascorbic acid the acidic reaction arises rather from the corresponding enol-lactone.

Another point which should be discussed is the relationship between the methylene blue-reducing compound formed at pH 7 during the irreversible transformation (3) and the reducing compound reported in this work. In the description given by Borsook *et al.* it is implied that a single transformation product has acquired this property. As information is lacking as to how much of this methylene blue-reducing product has been formed, it is premature to state to which product of the irreversible reaction the methylene blue-reducing property should be ascribed.

As regards the fate of vitamin C in the animal body, it can be assumed that its metabolic breakdown starts with the irreversible transformation of the dehydro vitamin. Since the stationary concentration of dehydroascorbic acid must be expected to be but a fraction of the actual vitamin C concentration in living tissue, owing to the natural protective system for this vitamin and particularly to the regular presence of excessive glutathione (12),[4] the mechanism of degradation follows Equation 2 mainly, with the quantitative formation of oxalic acid.

The quantitative formation of oxalic acid as part of the biological degradation of ascorbic acid suggests a possible relationship between the

[4] Particularly Table X, p. 263 (12).

vitamin C metabolism and the normal excretion of oxalic acid in the urine. Vitamin C would thus be the natural precursor of excreted oxalate. This assumption is in full agreement both with the independence of oxalate metabolism of food intake and the molecular relationship between the normal vitamin C requirement and oxalate excretion. According to recent reliable figures (13) the daily urinary excretion of oxalic acid of men amounts on an average to 27 mg., corresponding to the daily requirements of 50 mg. of ascorbic acid.

SUMMARY

1. The product of the irreversible and non-oxidative transformation of dehydroascorbic acid at pH 7 and in the presence of phosphate buffer is not diketogulonic acid or any other derivative of dehydroascorbic acid having an intact 6-carbon chain.

2. Under the above experimental conditions dehydroascorbic acid, in its stabilized form, probably as enolized diketogulonic acid lactone, suffers a scission of the 6-carbon chain with the quantitative formation of oxalic acid.

3. In the presence of oxygen, the oxalic acid formation from dehydroascorbic acid is preceded by the formation of an oxalyl compound which is most likely oxalylthreonic acid.

4. The presence of phosphate is obligatory for the irreversible transformation at pH 7. Phosphate causes catalytically the spontaneous formation of oxalic acid. In the absence of phosphate the 6-carbon chain remains intact, thus confirming the findings of Herbert, Hirst, *et al.* at hydrion concentrations below and slightly above pH 7.

5. The catalytic action of phosphate can be effectively replaced by cyanide.

6. Parallel with the irreversible transformation, dehydroascorbic acid suffers a dismutative change. This change, though accelerated both by phosphate and cyanide, does not depend upon the presence of these compounds. The products of the dismutative change are (1) a reducing compound, the reducing power of which persisted in acid solution and showed qualitatively the absorption characteristics of ascorbic acid in the ultraviolet region, and (2) an oxidized derivative of dehydroascorbic acid. The latter compound is characterized by its intense yellow color at pH 7 ($\lambda_{max.}$ 410 mμ). The dismutative change is of no importance at dehydroascorbic acid concentrations prevailing in animal tissues; it assumes measurable proportions only at concentrations $> 1 \times 10^{-3}$ M.

7. The biological significance of the irreversible transformation is discussed.

The author wishes to express his thanks to his colleague Dr. F. Goldschmidt who kindly carried out the spectroscopic tests in the ultraviolet region. Thanks are also due to Dr. T. Berlin for his valuable support in carrying out the simultaneous analytical tests.

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AMINO ACID NUTRITION OF LACTOBACILLUS ARABINOSUS

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The growth factors required by *Lactobacillus arabinosus* 17-5 have been studied extensively by Snell and Wright (1), Isbell (2), and Lewis (3). Investigation of the nitrogen requirement has been limited. Hydrolyzed casein has been found to supply the essential nitrogen when supplemented with tryptophane and cystine. Glutamic acid has been found essential (4), and quantitative levels have been determined. Recently, the amino acids essential for the growth of *Lactobacillus casei* (5) have been determined qualitatively.

The present investigation of the nitrogen requirements of *Lactobacillus arabinosus* has led to the discovery of the several amino acids necessary for the growth of this bacterium.

Method

Pollack and Lindner (4) have shown that a synthetic medium composed of twenty amino acids, salts, dextrose, sodium acetate, vitamins, and adenine, guanine, and uracil will support growth of *Lactobacillus arabinosus*. Hence preliminary experiments were carried out in a medium similar to that of Pollack and Lindner, with the exception that a different amino acid was omitted in each run. Lack of growth as shown by turbidity and acid production was taken as evidence of the essential character of the amino acid. Subsequent experiments were conducted to determine the level at which the amino acid promoted growth. Modifications were made in the amino acid content of the medium as levels were determined. The medium presented in Table I gave the best growth in the first series of experiments. Medium *b*, obtained after completion of the first series, gave theoretical acid production.

EXPERIMENTAL

Snell[1] has shown that at least two strains of the original culture[2] of *Lactobacillus arabinosus* exist: one needs *p*-aminobenzoic acid; the other, which is a mutant, does not. However, this mutant seems to be similar in all other characteristics to the original strain. The bacterium used in this study was the mutant as determined by its growth in the *p*-aminobenzoic acid-free medium of Lewis (3).

[1] Snell, E. E., private communication.
[2] American Type Culture Collection, No. 8014.

Stab cultures of the organism were carried on yeast extract-dextrose-agar (Difco), and were subcultured monthly. After transfer, cultures were incubated at 30° for 24 to 48 hours, and were then held in the refrigerator. Inoculum for assay tubes was prepared by transfer from a stock culture to a sterile centrifuge tube of the basal medium supplemented with the appropriate amino acid. The inoculum was incubated at 30° for 24 hours before use.

The basal medium had the composition shown in Table I. The amino acid for which levels were to be determined was omitted from the basal medium.

TABLE I

Basal Medium

	per cent		p.p.m.
Glucose	1	$l(-)$-Leucine†	50
Sodium acetate	0.6	dl-Isoleucine†	50
	p.p.m.	dl-Valine†	50
Adenine	10	$l(-)$-Cystine†	10
Guanine	10	dl-Methionine†	20
Uracil	10	$l(-)$-Tryptophane†	10
Thiamine	0.1	$l(-)$-Tyrosine	10
Riboflavin	0.2	dl-β-Phenylalanine	20
Nicotinic acid	0.2	$l(+)$-Glutamic acid†	200
Biotin	0.0004	dl-Threonine†	100
Pyridoxine	0.1	dl-Alanine	200
Pantothenic acid	0.1	$l(-)$-Asparagine or aspartic acid	160
p-Aminobenzoic acid*	0.01	$l(+)$-Lysine	40
Salts A and B (see text)		$l(+)$-Arginine	10

* p-Aminobenzoic acid was introduced into the medium before receipt of Snell's communication. The bacterium was checked for requirement of p-aminobenzoic acid before and after these experiments.

† Essential for *Lactobacillus arabinosus*.

Salts A and B were those of Snell and Wright (1). To Salts B were added a few drops of concentrated hydrochloric acid to prevent precipitation. 0.5 ml. each of Salts A and B was used for each ten test-tubes.

Adenine, guanine, and uracil,[3] thiamine, calcium pantothenate, riboflavin, and pyridoxine stock solutions were prepared and stored as directed by Snell and Wright (1). Nicotinic acid and biotin[4] were made up in 50 per cent ethanol to prevent bacterial decomposition. p-Aminobenzoic acid was prepared in glacial acetic acid and stored in amber ware.

[3] Eastman Kodak Company products.
[4] S. M. A. Corporation concentrate, No. 5000.

Procedure

Experiments were performed according to accepted microbiological procedure (1, 6). The amino acids[5] were weighed individually for each run and were dissolved in distilled water or a few ml. of hydrochloric acid. They were then made up to a convenient predetermined volume. The media, which were made double the final concentration, were adjusted to pH 6.6 to 6.8 with 1 N sodium hydroxide and diluted so that there were 5 ml. of medium per test-tube. 1 to 5 ml. of the standard amino acid solutions, neutralized to pH 6.8, was added to the tubes at ten levels chosen from preliminary curves. For lysine, for example, these were 0, 20, 40, 60, 80, 100, 120, 160, 240, 400 γ per test-tube. Each was diluted with 5 ml. of the basal medium and sufficient water to make a total volume of 10 ml. The tubes were plugged with cotton and sterilized by autoclaving 15 minutes at 15 pounds pressure. After cooling to room temperature, they were inoculated. All tubes were set up in duplicate for each experiment.

The cells from a 24 hour culture of inoculum grown in a 15 ml. Pyrex centrifuge tube were centrifuged aseptically and then washed two to four times by suspension in sterile 0.9 per cent saline and centrifuging. The bacteria were finally suspended in 30 ml. of saline. 1 drop (0.03 ml.) was used per test-tube. Aseptic precautions were observed. The tubes were incubated at 30° for 72 hours.

Measurement of Response—Titration of the lactic acid produced was employed as a measure of response. Brom-thymol blue was a satisfactory indicator. Titrations were reproducible to 0.1 ml. of 0.1 N sodium hydroxide.

Results

Results of several series of experiments at two different concentrations of amino acids in the basal medium are presented in Table II. These results indicate that the amino acids may be divided into three groups with respect to their action on the growth of *Lactobacillus arabinosus;* namely, essential, non-essential, and auxiliary. Auxiliary amino acids include tyrosine and phenylalanine, which are found essential with Medium *a* but not with Medium *b*.

Curves are presented for tryptophane (Fig. 1) and arginine (Fig. 2). Curve A (Fig. 1) was obtained with a hydrolyzed casein medium and a tryptophane supplement. The excellent agreement between Curves A and B, which was obtained with the medium presented in Table I, indicates that

[5] Amino acids were of C.P. or analytically pure grade of Amino Acid Manufactures, University of California at Los Angeles. Threonine, tryptophane, and cystine were from Merck and Company, Inc.

the factor limiting growth in Curve B is tryptophane and not the total amount of amino acids. Arginine is unique. The curve presented was obtained on five occasions. Experiments on this effect were not made with Medium *b*.

TABLE II

Effect of Amino Acids on Growth of Lactobacillus arabinosus

Amino acid	Concentration in basal Medium *a*	0.1 N acid formed when each is omitted*	Concentration at growth		Concetration in basal Medium *b*†	Concentration at half maximum growth	Concentration at maximum growth
			Half maximum	Maximum			
	p.p.m.	ml.	p.p.m.	p.p.m.	p.p.m.	p.p.m.	p.p.m.
Glutamic acid......	200	0.8	20	80	400	12	80
Threonine.........	100	1.2	8	40	200	10	30
Leucine...........	50	0.5	2	8	200	3.5	12
Isoleucine.........	50	0.3	3	10	200	5	20
Valine.............	50	0.3	3.5	10	200	6	16
Methionine........	20	1.1	2.5	10	100	3.2	12
Cystine...........	10	0.8	0.5	2.0	100	2.0	8.0
Tryptophane......	10	0.4	0.4	1.6	33	0.6	2.0
Tyrosine‡.........	10	0.8	0.12	0.5	33		
Phenylalanine‡.....	20	1.1	3.0	12.0	100		
Lysine.............	40	3.0	8	40	200		
dl-Alanine.........	200	4.0	20	80	200		
Arginine§..........	10	3.0	(See Fig. 2)	10	50		
Asparagine‖........	160	3.3	40	200	400		
Histidine ¶........		7.0			50		
Glycine**.........		7.2					
Serine**...........		5.8					
Proline**..........		6.1					
Hydroxyproline**..		7.7					

* The acid production when no amino acid of the above is omitted was 7.1 ml.

† The maximal acid production for basal Medium *b* varied from 8 to 11 ml. of 0.1 N acid.

‡ Tyrosine and phenylalanine could not be shown essential for *Lactobacillus arabinosus* on basal Medium *b*.

§ Arginine has three values for half maximal acid production. This is shown in Fig. 2. The acid production when arginine was omitted varied considerably.

‖ Asparagine and aspartic acid could be substituted for each other.

¶ Histidine increased acid production at a level of 10 to 100 p.p.m.

** Omitted from basal Media *a* and *b*. Each was assayed at 100 p.p.m.

Experiments with basal Medium *b* increased the acid production of 6 to 8 ml. of 0.1 N acid to 8 to 11 ml. Theoretical production is 11.0 ml. Glycine, serine, proline, and hydroxyproline were completely without effect on Medium *a* at levels up to 5 mg. per test-tube. Histidine at levels of from

10 to 100 parts per million raised 72 hour acid production with Medium a from 7.5 to 8.5 ml. and 84 hour production from 8.5 to 9.5 ml. Efforts to increase growth in Medium a by increasing glutamic acid to 10 mg. per tube caused slight inhibition of growth.
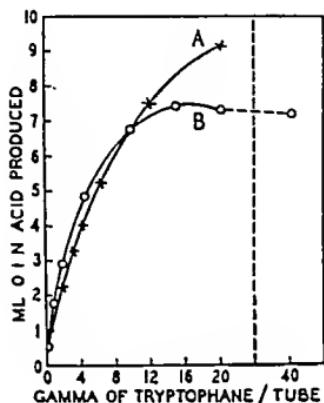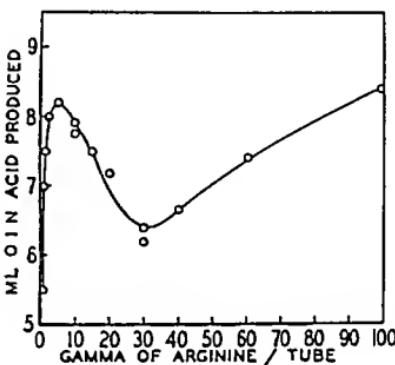
FIG. 1.   FIG. 2.

FIG. 1. Response of *Lactobacillus arabinosus* 17-5 to added tryptophane. The dotted line indicates change of scale in the abscissa. The points plotted are the average of two observed values. All curves were obtained on two separate occasions. Curve A, hydrolyzed casein medium; Curve B, synthetic amino acid medium.

FIG. 2. Response of *Lactobacillus arabinosus* 17-5 to added arginine. The points plotted are the average of two observed values.

DISCUSSION

Hutchings and Peterson (5) have shown an inhibitory effect of amino acids on the growth of *Lactobacillus casei*. Of those amino acids studied here, arginine showed one level of inhibition and two of stimulation. This is quite distinct from the work on *Lactobacillus casei*, in which the effect concerned the reciprocal effects of several amino acids and not different levels of the same acid.

Theoretical acid production was obtained with the isoleucine, leucine, and valine series with basal Medium b. Hence these experiments complete the study of factors essential for the growth of *Lactobacillus arabinosus*. It does not eliminate substances which may cause more rapid growth.

Assays of unknown amino acid mixtures have been made by the above techniques and will be reported elsewhere.

SUMMARY

Cystine, methionine, tryptophane, leucine, isoleucine, valine, glutamic acid, and threonine have been shown essential nutrilites for *Lactobacillus arabinosus*. Quantitative requirements have been determined.

A new effect of an amino acid on growth has been shown; namely, two levels of stimulation for arginine with an intermediate range of inhibition.

Thanks are due Miss D. Johnson for assistance with the manuscript.

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A PHOTOMETRIC MICROMETHOD FOR THE DETERMINATION OF INULIN IN SERUM AND URINE

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The application of the inulin clearance test to infants and children has been held back by lack of a quantitative method for inulin which requires only small samples of blood.

The technique of Alving and coworkers (1), based upon the hydrolysis of inulin to fructose and the subsequent reaction of the latter with diphenylamine, is widely used in adults. Harrison (2) modified this procedure by substituting a less volatile liquid, glacial acetic acid, for ethanol as the solvent of the diphenylamine; he also reduced the required volume of serum from 5.0 to 0.2 cc. However, diphenylamine yields considerable color with glucose; hence glucose must always be removed from the serum and sometimes from the urine by treatment with yeast before inulin can be determined. More recently Hubbard and Loomis (3) have applied Seliwanoff's reaction, specific for fructose, to the measurement of inulin. While this method eliminates the need for fermentation, it is less sensitive than Harrison's technique for the diphenylamine reaction and requires a larger sample.

Jordan and Pryde (4) noted that a color is formed by the interaction of fructose and skatole under proper conditions. Reinecke (5) adapted this reaction to the quantitative measurement of fructose in the blood. We have applied Reinecke's procedure for fructose to the quantitative determination of inulin in serum and urine.

Method

To 5.0 cc. of dilute tungstic acid 0.2 cc. of serum is added with a calibrated Hagedorn-Jensen blood pipette. The proteins are separated by centrifuging and 2.0 cc. of the supernatant liquid are transferred to each of two Bailey-Myers sugar tubes calibrated at 10 cc. To each are then added 4 cc. of saturated ethanolic hydrogen chloride. The contents are stirred with a glass rod which is left in the tube. The mouth is closed loosely with a glass sphere and the tubes are heated for 30 minutes in a water bath at 60°, then cooled for 2 to 4 minutes in an ice bath. To each is added 0.1 cc. of 1 per cent skatole dissolved in 95 per cent ethanol. Between 5 and 7 minutes later the contents are stirred and diluted to 10 cc. with 95 per

THE COLORIMETRIC DETERMINATION OF CHOLESTEROL

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It is widely recognized that most of the methods in general use for the determination of cholesterol, based on the Liebermann-Burchard color reaction, are unsatisfactory, and the principal sources of inaccuracy are known; but it does not appear to be realized that large errors may result from relatively small variations in procedure. Recently we were stimulated to investigate the colorimetric estimation of cholesterol by a request that we recommend a procedure for inclusion in the "Technical manual of methods for laboratory technicians of the War Department." Over 550 known and unknown samples were read under various conditions. Some of the findings are presented here primarily to direct attention to the potential inaccuracies in cholesterol methods as they are ordinarily used. A procedure with which good analyses can be obtained will be summarized and the sources of error will then be discussed in relation to the steps of that procedure.

Procedure

Pipette 0.4 cc. of blood serum into approximately 5 cc. of alcohol-acetone (1:1) in a 10 cc. volumetric flask (or 1 cc. of serum in a 25 cc. flask if replicate determinations are desired) with swirling of the solution, bring the solvent to a boil on the steam bath, cool the flask, make up to the mark with alcohol-acetone, mix, filter, and pipette 5 cc. of the filtrate into a 25 cc. Erlenmeyer flask to which 0.15 cc. of potassium hydroxide solution (10 gm. in 20 cc. of water) has previously been added. Swirl the liquid gently at intervals until the alkali has mixed completely with the alcohol-acetone, place the flask in an incubator at 37–40° for 40 minutes, add 1 drop of phenolphthalein solution, titrate with 10 per cent acetic acid in absolute alcohol (about 0.6 cc. should be required[1]), add 1 drop excess, and evaporate just to dryness on the steam bath with the aid of a stream of air applied by means of suction through a glass tube, bent to avoid contamination from the rubber connection and clamped about 2 cm. above the surface of the solution. Cool the flask, add 0.1 cc. of 50 per cent alcohol without delay,

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[1] The solution of acetic acid slowly loses strength through the formation of ethyl acetate.

and wash down the walls of the flask with about 3 cc. of petroleum ether. Swirl gently at intervals until the salt dissolves and a clear separation of the two liquids occurs. If the salt does not dissolve completely within 10 minutes, add 0.05 cc. more 50 per cent alcohol. Decant the petroleum ether through a small funnel into a dry $\frac{1}{2}$ or 1 ounce bottle, equipped with a well fitting glass stopper, with care that none of the aqueous layer is carried over. Repeat the washing with small portions of petroleum ether five times more and evaporate the combined extracts to *complete dryness* by placing the bottle in a small beaker of cold water which is heated on the steam bath while a stream of air is applied by means of a suction line as before. If there is any doubt that the residue is dry, add a few drops of absolute alcohol, rotate the bottle so the walls are wet, and repeat the drying process.

Development of Color and Reading—Adjust to 24° a water bath placed in a dark cabinet (a packing box, equipped with a door, will serve) and maintain the temperature at this point throughout the development of color by adding hot or cold water as needed. Pipette 5 cc. of acid-free chloroform into each bottle containing dried serum extract and 5 cc. portions of standard solutions containing 0.24, 0.4, and 0.6 mg. of pure cholesterol in chloroform into three similar bottles. Stopper and place the bottles in the water bath, using a support, such as a wire basket, so that they will not tip over. Measure 20 cc. of pure acetic anhydride into a suitable glass-stoppered container and chill in an ice bath. Add 1 cc. of concentrated sulfuric acid with shaking while the container is kept in the ice bath.[2] Start a stopwatch. At about 9 minutes remove one of the standard samples from the 24° bath, wipe the bottle dry, add 2 cc. of the cold reagent, shake the bottle for 10 seconds, and return it to the bath. The time of starting this process will depend on the time it requires (speed of the pipette, etc.); it should be so timed that the bottle is returned to the bath at 10 minutes ±5 seconds. The interval between samples will be determined by the time required for making a reading, to be established by practice. The color must be developed in all samples with exactly the same technique and with the same timing. Read in a colorimeter between 17 and 18 minutes after the bottle is returned to the bath against a solution containing 14 mg. of naphthol green B per 100 cc. The dye solution is standardized against each of the three standard cholesterol solutions and the unknown solutions are read against the "standard setting" of the dye with which they are in closest agreement. If a light filter with maximal transmission at about 625 $m\mu$ is

[2] This mixed reagent is now being used in the method of Schoenheimer and Sperry (1). A description of this procedure with some modifications (2, 3), which have been adopted since the original publication, is being prepared and will be available on request.

available, an ink standard as described by Shapiro, Lerner, and Posen (4) or neutral gray filters may be used in place of the dye solution.

The degree of variation among replicate determinations carried out with this procedure on 50 cc. extracts of 2 cc. portions of serum is shown in Table

TABLE I

Variation among Replicate Determinations by Proposed Method

2 cc. portions of serum were extracted in 50 cc. volumetric flasks. 5 cc. portions of the extracts were analyzed by the proposed method and 1 cc. portions by a modification of the method of Schoenheimer and Sperry (1). All sera, except Sera 7, 8, 10, 13, and 15, were pooled samples.

Serum No.	Proposed method		Method of Schoen-heimer and Sperry
	Replicate determinations	Average	
	mg. per 100 cc.	*mg. per 100 cc.*	*mg. per 100 cc.*
1	232, 228, 233, 228, 234, 230, 231, 232	231	233
2	230, 233, 234, 231, 231, 234, 230, 231	232	230
3	227, 206* 228, 227, 226, 228, 234, 235	229	228
4	231, 233, 231, 242, 237, 234, 240, 229	235	236
5	221, 218, 221, 222	221	219
6	226, 217, 222, 213	220	223
7	242, 244, 241, 249, 245, 244, 239	243	240
8	184, 183, 185, 185	184	181
9	233, 230, 233, 233	232	235
10	180, 178, 180, 182, 180	180	176
11	256, 250, 240, 256, 243	249	246
12	220, 227, 227, 217	223	217
13	220, 219, 218, 216	218	214
14	238, 234, 239, 235	237	240
15	216, 216, 221, 219	218	223

* Probably not dry; omitted from the average.

I. 1 cc. portions of the same extracts were analyzed by a modification[2] of the method of Schoenheimer and Sperry.[3]

[3] Dimter (5) reported recently that a substance which gives a positive Liebermann-Burchard reaction, but which is not precipitated by digitonin, is present in the unsaponifiable fraction of human serum. The close agreement (Table I) of values yielded by a method in which the color reaction is applied directly with those obtained with a procedure in which the cholesterol is first isolated as the digitonide is not in accord with this finding unless the substance occurs in very small quantity, within the limits of error of the methods. Dimter gives no information concerning the quantity of the unknown substance, or substances, in serum, though he implies that it is present in considerable amount. In one of his fractions, the Liebermann-Burchard reaction developed very slowly and it is possible that the color had not yet developed under the conditions employed in the present investigation.

DISCUSSION

Necessity of Saponification—In 1917 Bloor (6) reported that the Lieber-mann-Burchard color developed more rapidly with blood extracts than with pure cholesterol. The difference became larger as the temperature was decreased. A hint at the reason for this effect was given by Gardner and Williams (7) in their incidental observation that cholesterol benzoate and acetate gave values which were 5 and 11 per cent, respectively, too high with the colorimetric procedure. Many years later the explanation became clear with the finding, made independently by Reinhold (8), Yasuda (9), Noyons (10), and Kelsey (11), that esterified cholesterol develops color at a more rapid rate, or to a greater extent, than does free cholesterol. The difference was found by Reinhold to be so large at 0–2° as to permit the differential estimation of combined cholesterol. Kelsey reported an experiment in which he measured the course of color development in solutions of cholesterol and of cholesterol palmitate at 23°. With this exception, no systematic study of the magnitude of the effect under the conditions ordinarily employed for cholesterol determination has been reported.

In a series of experiments the color was developed with the technique described above, or in some cases with separate addition of acetic anhydride and sulfuric acid, in solutions of recrystallized cholesterol (m.p. 147.5–148°, uncorrected) and of cholesterol palmitate (m.p. 78–79°, uncorrected), containing equivalent quantities of cholesterol. Sufficiently large volumes were employed so that a series of readings could be carried out on the same solution over a period of time. Portions were withdrawn with a transfer pipette without removing the solutions from the bath. In each experiment the cholesterol and the ester were carried through side by side under conditions as nearly identical as could be maintained. The two solutions were read alternately in a photoelectric photometer (12) with Corning light filters No. 244 and No. 978. Typical results of two such experiments are shown in Fig. 1. It will be seen that there was a considerable difference between cholesterol and the palmitate, not only in the density of color at the maximum, but also in the rate of color development. With the ester more color was produced and the maximum was reached earlier than with cholesterol. The findings are in general agreement with those of previous investigators, but they differ in detail from the results reported by Kelsey (11) who found that the curves yielded by cholesterol and its palmitate at 23° were approximately parallel, with a maximum at about 15 minutes in each case, and that the difference between maxima was about 30 per cent. In accord with the results of Bloor (6) and of Reinhold (8) our experiments show a considerable temperature effect. The percentage difference be-

tween maxima was about the same at 20° and at 24°, but there was a much larger difference in the time at which the maximum was reached at the lower temperature.

Since in most colorimetric methods in general use a saponification step is not included, and since blood serum contains a high proportion of cholesterol esters, this factor represents a source of considerable error and, as stressed by Kelsey (11), probably accounts, at least in part, for the high values usually obtained by these methods. It is possible that the unsaturated esters which characterize blood serum (13) may not behave like
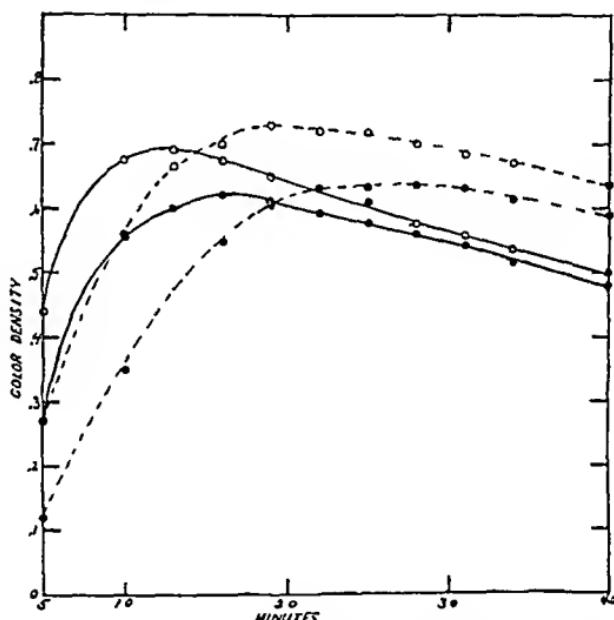
FIG. 1. Curves showing the rate of color development in solutions containing equivalent quantities of cholesterol in the free and combined forms. ● free cholesterol; ○ cholesterol palmitate; solid lines, color developed at 24°; broken lines, color developed at 20°.

the palmitate, but there is strong evidence against this assumption: (a) the observations reported by Bloor (6); (b) the findings by Noyons (10) of 17 per cent lower values with the digitonin method than with the colorimetric method applied to unsaponified serum extracts, and of 24 per cent lower values following saponification than without saponification with the colorimetric method; and (c) Yasuda's report (9) that the oleate gives about the same high result as the palmitate and stearate.

Extraneous Color—Most workers with colorimetric methods for cholesterol determination have been troubled by off shade colors, occurring more or less at random. In recent years the difficulty has been overcome to a

considerable extent by the use of filters which mask out the extraneous yellow or brown colors. We have carried out no systematic study of this factor, but some observations are pertinent. In one instance a series of samples was allowed to stand overnight at room temperature after incubation without acidification. A brownish yellow color developed in all samples. With the dry extraction procedure then in use some of this color was carried into the final chloroform solutions and it was difficult or impossible to obtain satisfactory readings. Bloor, Pelkan, and Allen (14) called attention to the danger of heating with strong alkali. For this reason we recommend the use of the mild conditions for saponification employed in the method of Schoenheimer and Sperry (1), and acidification without delay.

During the early part of this study an alcoholic solution of hydrochloric acid was used for acidification. In some cases a brown color was seen to develop during evaporation just before dryness was reached and almost immediately the solvent refluxing in the suction tube became deeply yellow, while the color in the residue decreased. Apparently some partially volatile pigment was formed by action of hydrochloric acid. This difficulty was avoided by the use of acetic acid.

With the method as described no trouble with extraneous colors has been experienced. We attribute this result not only to the factors mentioned, but also to the wet extraction procedure. Fairly good results were obtained by extracting the dry residue with petroleum ether or chloroform, but off shade colors were sometimes encountered and the values were more variable than those obtained with extraction from water and alcohol. The yellow color which is usually present appears to be retained in the aqueous alcohol layer.

Extraction after Saponification—After the development of color during evaporation in the presence of hydrochloric acid was discovered, two procedures of neutralization were investigated: (a) titration with hydrochloric acid followed by alkalization with a drop or two of dilute potassium hydroxide solution, and (b) titration with acetic acid. In addition in some experiments sodium hydroxide was used as the saponifying agent and titrated with acetic acid. All of these procedures were studied in conjunction with dry extraction with various solvents (petroleum ether and chloroform for the most part) and with petroleum ether extraction after the addition of water. Aside from the variation already noted dry extraction has the disadvantage of requiring filtration. Petroleum ether extracts from samples to which water alone was added tended to be cloudy and the results were usually low. The addition of alcohol facilitates the separation; it may be used under the conditions described because potassium acetate is very soluble in alcohol. This salt has the disadvantage of being deliquescent, but this gives no trouble if the extraction is carried out immediately after drying.

Development of Color. (a) *Method of Adding Reagents*—In practically all methods for the colorimetric estimation of cholesterol acetic anhydride and sulfuric acid are added separately to the chloroform solution. It is difficult to measure accurately the small quantity of sulfuric acid and this has been recognized as a potential source of error. Furthermore, since heat is produced rapidly after addition of the acid, and since the development of color is greatly affected by temperature, variations in the amount of acid, in the speed of addition, and in the speed of mixing may have a marked effect on the final result. That this is the case is indicated by the data presented in Fig. 2. Equal volumes of a solution of cholesterol in chloroform were placed in glass-stoppered cylinders such as are used in most methods for the development of color. After the temperature had been adjusted to 24°, acetic anhydride was added and mixed well with the chloroform. Sulfuric acid was then added from a micro burette, as described by Schoenheimer and Sperry (1), the solution was mixed thoroughly, and the cylinder was returned to the bath. Samples were read in the photoelectric photometer at the times after addition of the acid indicated in Fig. 2. Although every attempt was made to maintain exactly the same conditions throughout the procedure, in only two of the eight samples did the curves of color development coincide almost exactly. At the extreme there was a difference of 11 per cent between maxima. We are convinced from the foregoing experiments and from much experience with the Liebermann-Burchard reaction that the whole course of color development and the density of color produced at the maximum are determined, other factors being constant, by the events occurring immediately after the addition of sulfuric acid.

To avoid these difficulties Shapiro, Lerner, and Posen (4) employed a mixture of acetic anhydride and sulfuric acid. These substances react rapidly to form acetyl sulfuric acid with the evolution of considerable heat, and this reaction is followed by the much slower formation of sulfoacetic acid (15). Shapiro *et al.* cautioned that the mixed reagent is stable for 1 hour only. With a reagent mixed at room temperature according to their directions we obtained constant readings up to 35 minutes after mixing, but there was evidence of some change at 45 minutes (Table II). Constant results were obtained up to 1 hour after mixing with a reagent mixed and kept at ice bath temperature (Table II), and in other experiments only a small change was observed at 110 minutes under these conditions. With this reagent satisfactorily consistent results have been uniformly obtained.[2]

(b) *Effect of Temperature*—Although it has been known for many years that the development of color in the Liebermann-Burchard reaction is influenced by temperature (6), in most methods little attention is paid to the control of this variable. Ranges of 20–25° are specified in some procedures. An indication of the variation which may occur within this range

may be obtained by comparing the curves for free cholesterol at 20° and 24° in Fig. 1. The color developed much more slowly at the lower temperature
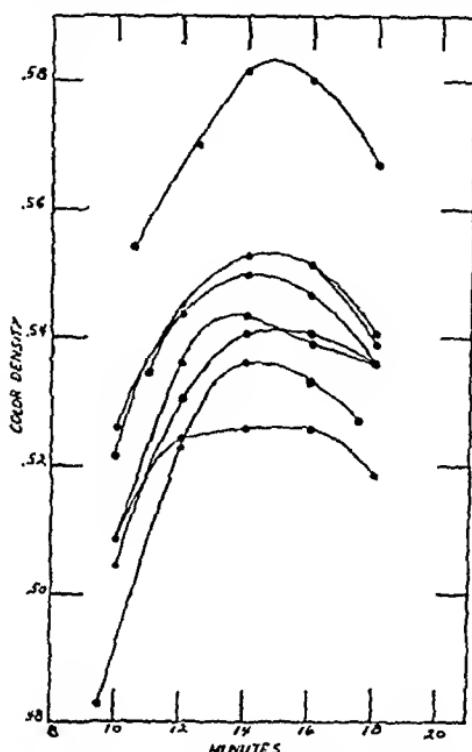
Fig. 2. Variation in development of color in the Liebermann-Burchard reaction with separate addition of acetic anhydride and sulfuric acid. All samples contained the same amount of cholesterol and were treated under conditions as nearly identical as possible.

TABLE II

Stability of Acetic Anhydride-Sulfuric Acid Reagent

The data represent color density as read in a photoelectric photometer.

Time of reading after adding reagent	Reagent mixed at room temperature				Time of reading after adding reagent	Reagent mixed in ice bath			
	Time after mixing reagent					Time after mixing reagent			
	15 min.	25 min.	35 min.	45 min.		5 min.	20 min.	40 min.	60 min.
min.					*min.*				
12	0.276	0.261	0.265	0.255	12	0.640	0.644	0.653	0.654
14	0.326	0.319	0.316	0.287	18	0.602	0.611	0.602	0.606
16	0.368	0.368	0.367	0.352	24	0.553	0.562	0.561	0.561
18	0.415		0.409	0.389	30	0.509	0.523	0.520	0.520
20	0.444	0.446	0.439	0.425					

and reached a higher maximum. Except at the point where the curves cross (19 minutes) comparisons between two solutions in which color was developed at these two temperatures would be grossly in error. When the acetic anhydride and sulfuric acid are added separately, the effect of temperature may be even greater; in one experiment a difference of 35 per cent between the maxima developed at 20° and 25° was observed.

It may be argued that the effect of temperature is not important because standards and unknowns are treated side by side under the same conditions. This is true to a certain extent if a bath is used, but when the containers are allowed to stand in the air during color development, as in many methods, there is no assurance that the temperature is the same for all samples. The development of heat at the start may vary, as already pointed out, and the rate at which the heat is dissipated may vary with the thickness of the glass walls of the containers and perhaps with other factors. In some methods the use of a bath is recommended, but no particular temperature is specified. This is not sufficient, because for good results the temperature and time must be so controlled that the reading is taken at the point of maximal density where the rate of change is at a minimum. Measurements during the period when color is developing rapidly are particularly subject to error (*cf.* Fig. 1).

(c) *Effect of Time*—The importance of developing the color for the same length of time in all samples is apparent from the foregoing discussion. The common practice of reading several unknown samples against a single standard cholesterol solution introduces a large potential source of error, since the color of the standard is changing during the time the readings are carried out.

It will be noted (Fig. 1) that a much broader maximum is obtained at 20° than at 24°. By working at the lower temperature considerably more latitude in timing would be permissible. However, it is difficult to maintain a bath at 20° during warm weather and for this reason we selected 24°. At higher temperatures the color develops too rapidly.

Brown Color—After several hours the green color of the Liebermann-Burchard reaction fades out and a yellowish brown color supervenes. Whereas the development of green color is entirely empirical and is influenced by several factors as discussed above, it seemed possible that the density of the brown color, which appears to be an end-result of the reaction, might be proportional to the amount of cholesterol without regard to the conditions under which the color was developed. Hence in a number of the foregoing experiments and others not reported here the residual solution was retained overnight and the color was measured in the photoelectric photometer with filters having a maximal transmission in the violet, where the brown color shows maximal absorption. A wide variation

was found.   Moreover, it was impossible to read the color accurately in a colorimeter.

SUMMARY

Under the conditions ordinarily employed for the determination of cholesterol by means of the Liebermann-Burchard color reaction large errors may occur.

Saponification is necessary before the estimation of total cholesterol in blood serum or tissues containing esterified cholesterol, because in the combined form it develops considerably more color at a faster rate than does free cholesterol.

Interference by extraneous colors may be avoided by the use of mild conditions for saponification and a weak acid (acetic) for acidification, and by extraction from a water-alcohol solution of the acidified residue.

The development of color may be influenced to a considerable extent by small and uncontrollable variations in the procedure of adding acetic anhydride and sulfuric acid.   This source of error may be avoided by mixing these reagents beforehand (4).

Small variations in the temperature at which color is developed have a large effect on the time at which the maximum is reached and on the height of the maximum.   The temperature and time of reading should be selected so that the reading is carried out at the maximum, and should be the same for each sample in a series of determinations.   The common practice of reading several unknown samples against one cholesterol standard violates this requirement.

A procedure which avoids these sources of error is described.

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A MICROBIOLOGICAL METHOD FOR THE DETERMINATION OF CHOLINE BY USE OF A MUTANT OF NEUROSPORA

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Previous communications from this laboratory have described the production of biochemical mutants in the mold *Neurospora* by means of ultraviolet and x-rays (1, 2). Such mutants are characterized by the inability to carry out specific chemical syntheses which normally occur in the unmutated, or wild type, strain. In each case which has been genetically analyzed the failure of the synthesis has been found to be related to the mutation of a single gene. The strain to be described, known as No. 34486, or *cholineless*, arose from a culture of wild type *Neurospora crassa* which had been irradiated with ultraviolet light. It was found to be unable to grow in a medium containing only salts, sugar, and biotin, but it grew normally on the addition of a mixture of water-soluble vitamins. When the components of the mixture were tested singly, it was found that the addition of choline alone permitted normal growth.

Up to the present, no completely satisfactory method for the determinaton of choline in natural products and tissue extracts has been described. Chemical methods, such as precipitation of the reineckate, lack specificity, while the biological method of Fletcher, Best, and Solandt (3) is time-consuming and difficult, and "possesses many dangerous pitfalls for the chemist" (4). The whole subject has been critically reviewed by Best and Lucas (4). It was therefore of interest to determine whether the *Neurospora* mutant is a suitable test organism in a quantitative assay for choline. The experiments to be described show that this is the case and form the basis of a simple, sensitive, and specific method for the determination of choline in natural products. By this procedure it is possible to determine choline in a concentration of 0.02 mg. per liter; routine analyses can be run on 100 mg. samples of material.

Methods

The basal medium used in these experiments has the following composition, in gm. per liter: ammonium tartrate 5, ammonium nitrate 1, monobasic potassium phosphate 1, magnesium sulfate ($7H_2O$) 0.5, sodium chloride 0.1, calcium chloride 0.1, sucrose 20, biotin 5×10^{-6}. In addition, it contains the following trace elements, added as salts, in mg. per liter: B 0.01, Mo 0.02, Fe 0.2, Cu 0.1, Mn 0.02, Zn 2.0. The medium is

made up without sucrose and biotin, is autoclaved, and is stored in 1 liter bottles. Sucrose and biotin are added before the medium is used. For convenience in storing, it may be made 3 times as concentrated as indicated and diluted before use.

Stock cultures of the mutant are maintained on agar slants composed of the basal medium plus the following: agar 1.5 per cent, Difco yeast extract 0.2 per cent, malt extract 0.2 per cent, choline 1 γ per ml.

In the assay for choline, the mold is grown in 250 ml. Erlenmeyer flasks containing 25 ml. of medium. To inoculate, a spore suspension is made up in a few ml. of sterile distilled water, and 1 drop of the suspension is added to each flask. The flasks are incubated at 25° for 3 days, at the end of which time the pads are removed, pressed out on filter paper, and dried at 90°. They are then weighed to the nearest half mg.

Results

Growth Studies—The growth rate of *cholineless* is a function of the concentration of choline in the medium. A typical growth response curve is shown in Fig. 1. The normal, *i.e.* wild type, growth rate is attained at a concentration of 50 to 60 γ of choline per 25 ml. Under the conditions of these experiments the weight of the pads after a 3 day growth period is practically independent of the size of the inoculum. Thus, at a concentration of 2 γ of choline per 25 ml. a 16-fold increase in inoculum size raised the dry weight from 12 to 16 mg.; at a level of 30 γ of choline no increase in dry weight occurred.

The specificity of the response was tested with a wide variety of compounds. Of the eleven water-soluble vitamins and twenty-two amino acids tested only choline and methionine were found to be active. Methionine is approximately 0.002 as active as choline. The activity is not increased by the simultaneous addition of ethanolamine. Lecithin was the only other compound found to be active. Using a sample of pure lecithin, we found that 50 per cent of the potentially available choline was utilized in a 3 day period. The following substances related to choline were inactive: betaine, creatine, sarcosine, ethanolamine, dimethylamine, trimethylamine, and tetramethylammonium chloride.

The maximum growth obtainable with methionine after 3 days is considerably below that reached with choline. This is shown in Fig. 2, the data for which were obtained with analytically pure, synthetic *dl*-methionine. This result is interpreted as indicating a sparing action of methionine on the small amount of choline present in the inoculum. Such an interpretation is in accord with the known relationship between choline and methionine in the rat (5, 6). By providing an extra source of labile methyl groups, the addition of methionine permits choline to be used for other

essential purposes, such as the synthesis of lecithin.   On this basis, it is to be expected that the effect of the simultaneous addition of methionine and choline (at a suboptimal level) will be greater than the sum of the individual effects.   This was found to be the case (Table I).   The most striking evidence for this conclusion is furnished by the growth of *cholineless* on agar medium in horizontal tubes.   In the presence of a given concentration of choline the rate of progression of the mycelial frontier, in mm. per hour, along the surface is a constant and continues at the constant rate to the end of the tube.   If, instead of choline, methionine is supplied, the rate
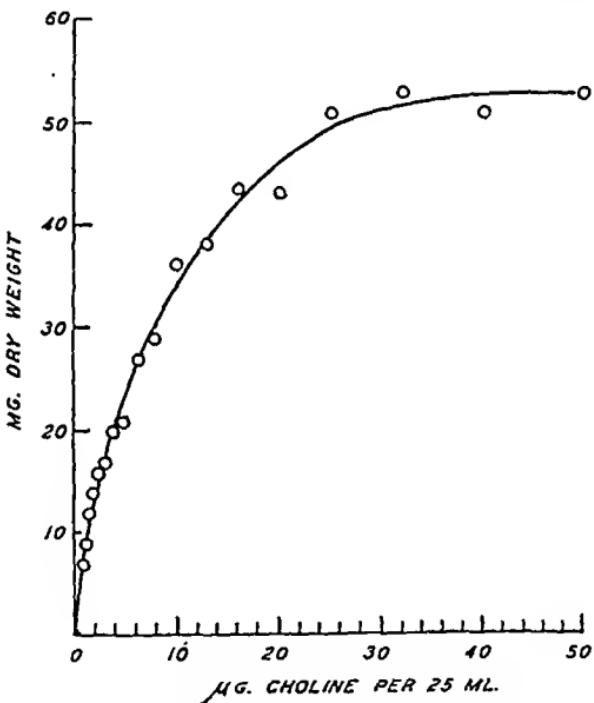
FIG. 1. Dry weight of *cholineless* after 3 days as a function of the concentration of choline in the medium.

first attains a characteristic value depending on the concentration of methionine and then falls off to zero before the end of the tube is reached.   This shows that methionine can replace choline in some, but not all, of its functions; as soon as the store of available choline in the inoculum is exhausted, growth ceases.   The methyl group of methionine is apparently not used for choline synthesis by the mutant.

Preparation of Materials for Assay—To assay natural products with *cholineless*, the sample is first autoclaved with 3 per cent sulfuric acid for 2 hours at 15 pounds.   This treatment liberates choline from lecithin which,

possibly because of its low solubility, is but slowly utilized by the mutant.
Table II shows that, in the materials tested, hydrolysis is complete within
2 hours and may be continued without loss of choline for at least 3.5 hours.
Refluxing with 3 per cent sulfuric acid for 7 hours gave essentially the same
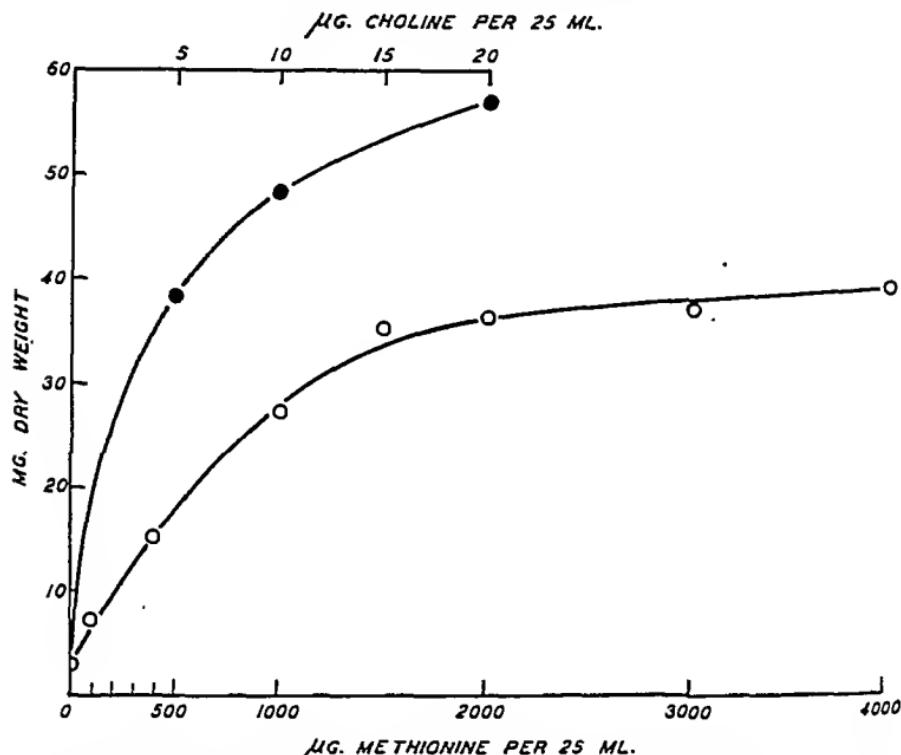
FIG. 2. Growth of *cholineless* on methionine after 3 days (open circles). The solid
circles show the growth curve on choline obtained simultaneously.

TABLE I

Effect of Simultaneous Addition of Choline and Methionine on Growth of Cholineless

Quantities are expressed in mg. of dry weight of mold after 70 hours at 25°.

Choline per 25 ml.	Mg. *dl*-methionine per 25 ml.			
	0	0.50	1.00	2.50
mg.				
0	0.5	7.0	10.5	30
0.002	17	34.5	46	62
0.030	60	61.5	64.5	75

result as autoclaving. Following neutralization with barium hydroxide,
the solution is treated with permutit[1] in order to separate choline from

[1] Permutit (according to Folin), obtained from The Coleman and Bell Company,
Norwood, Ohio.

methionine. *dl*-Methionine interferes with the choline assay when present in excess of 0.1 mg. per 25 ml. of culture medium. Although this concentration is fairly high, considering the conditions of mild hydrolysis and high dilution in the assay, it may be exceeded in those cases in which the material being tested contains much protein and little choline. In any case, the permutit treatment is recommended for the reason that it elimi-

TABLE II

Liberation of Choline from Natural Products by Various Treatments

100 mg. samples were hydrolyzed with 10 ml. of 3 per cent sulfuric acid by the procedures indicated. Choline content is expressed in micrograms per 100 mg.

Material	No treatment	Autoclaving					Refluxed 7 hrs.*
		1 hr.	1.75 hrs.	2.0 hrs.	2.5 hrs.	3.5 hrs.	
Dried brewers' yeast.....	48			262		258	266
" milk..............	60			81		83	88
White flour..............	16	120	125	119	132	130	138
Corn-meal...............	10	31	35	34	28	37	37

* The hydrolysates were not treated with permutit.

TABLE III

Elution of Choline from Permutit

5 ml. of a solution containing 20 γ of choline per ml. were passed through permutit columns, followed by 5 ml. of 0.3 per cent sodium chloride. Sodium chloride solutions in the amounts and concentrations indicated were then passed through and the filtrates assayed with *cholineless*.

Sodium chloride solution		Choline eluted
Concentration	Amount	
per cent	*ml.*	*per cent*
0.3	5	0
1.0	10	55
2.0	10	84
3.0	5	80
3.0	10	90
5.0	5	80
5.0	10	100

nates all non-basic substances which may inhibit or stimulate the growth of the mold.

The adsorption is carried out in columns measuring 110×0.6 mm., containing approximately 1 gm. of permutit. The design of these columns is given by Dubnoff and Borsook (7). The best conditions for adsorption and elution of choline were determined by running known solutions through

the columns and testing the filtrates with the mutant.   It was found that a permutit column of the above dimensions completely removes the choline from 5 ml. of a solution containing up to 0.5 mg. of choline per ml.   Repeated tests have shown that adsorbed choline is quantitatively eluted with 10 ml. of 5 per cent sodium chloride.   The results of eluting with various sodium chloride solutions are shown in Table III.

Methionine is not adsorbed by permutit.   Formol titrations made on solutions of methionine before and after passing through a permutit column, followed by washing with 0.3 per cent sodium chloride, gave quantitative recoveries.

Procedure

Details of the procedure which has been used in assaying for choline are as follows:

100 mg. of the dry material to be analyzed are weighed into a 50 ml. Erlenmeyer flask, followed by 10 ml. of 3 per cent sulfuric acid.   The flask is plugged with cotton and autoclaved at 15 to 17 pounds for 2 hours.

After cooling, the contents are transferred quantitatively to a 50 ml. Pyrex centrifuge tube and neutralized to Congo red with saturated barium hydroxide.   The barium sulfate, together with the undissolved residue remaining from the previous step, is centrifuged down, and the supernatant is filtered through a Whatman No. 50 paper.   3 ml. of distilled water are added to the precipitate in the centrifuge tube and the contents brought to a boil, with stirring.   After cooling and centrifuging, the washing is added to the previous supernatant.   The clear filtrate is neutralized to litmus with M sodium hydroxide.   It is then brought to a convenient volume, usually 30 ml., with distilled water.

5 ml. of the neutralized solution are run through a column of permutit of the dimensions described above.   If the solution is known to contain less than 3 γ of choline per ml., 10 ml. are usually run through.   The column is then washed with 5 ml. of 0.3 per cent sodium chloride.   The filtrate and washing are discarded.   A test-tube marked at 10 ml. is now placed under the column, and the choline is eluted with 10 ml. of 5 per cent sodium chloride.   The filtrate is brought to 10 ml. with distilled water. It is usually convenient to adsorb two or more portions of the solution simultaneously, in separate columns; in this way, sufficient filtrate is provided for an orienting assay in case the choline content is completely unknown.

The solution is distributed among 250 ml. Erlenmeyer flasks, and the volume in each flask is made up to 25 ml. with basal medium.   Usually not more than 5 ml. of the solution being tested is added to a flask.   For best accuracy, the final concentration of choline should lie between 0.5

and 20 γ per 25 ml. Each concentration of unknown is made in duplicate. At the same time a standard series is set up containing pure choline in a range of concentrations from 0 to 20 γ per flask. The flasks are autoclaved at 15 pounds for 5 to 10 minutes. After cooling, they are inoculated, placed in the incubator, and the dry weight of the mycelium determined at the end of 3 days. The choline values are calculated in the usual way from a plot of the standard series.

TABLE IV

Within-Series Reproducibility of Choline Assays and Recoveries of Added Choline

Material	Filtrate	Choline added	Duplicate dry weight ol mold	Choline found		Recovery of added choline
				Per flask	Per 100 mg. material	
	ml.	γ	mg.	γ	γ	γ
Dried brewers' yeast......	0.5	0	22, 23	2.2	264	
" " "	1.0	0	31, 31	4.3	258	
" " "	2.5	0	43, 44.5	11.0	264	
" " "	0.5	5.0	38, 40	7.2		5.0
" " "	2.5	5.0	47, 48	16.4		5.4
White flour..............	0.5	0	20.5, 22	2.0	120	
" "	1.0	0	28.5, 30.5	3.8	114	
" "	2.5	0	43, 43	10.2	122	
" "	0.5	5.0	38.5, 39	7.0		5.0
" "	2.0	5.0	46.5, 46.5	15.0		6.8
Corn-meal................	2.0	0	22.5, 23.5	2.3	34.5	
"	3.0	0	27.5, 28	3.4	34	
"	6.0	0	34.5, 35.5	5 5	27.5	
"	2.0	5.0	39.5, 40	7.5		5.2
"	6.0	5.0	41.5, 40.5	8.5		3.0
Dried milk................	1.0	0	24.5, 26.5	2.8	84	
" "	2.0	0	35.5, 36	5.8	87	
" "	5.0	0	43.5, 45.5	12.2	73	
" "	1.0	5.0	39.5, 40.5	7.6		4.8
" "	4.0	5.0	46, 47.5	15.4		4.6

By the above procedure, choline assays on ten different samples have been carried out simultaneously by one worker.

Reproducibility—Dry weights from duplicate flasks agree within 5 per cent, on the average. Choline values determined on different amounts of the same solution generally agree within 10 per cent. Recoveries of added choline are usually within 90 to 110 per cent of theoretical. Table IV shows the results obtained on four different products, all regular commercial samples.

It has been found that standard curves obtained on different days are

not, in general, superimposable. The variation is apparently not related to inoculum size, but possibly to the age and condition of the spores. This does not affect assay values, which show good day to day reproducibility. It is necessary, however, to run a standard series each time a new spore suspension is used.

In Table V is shown the choline content of a number of different natural products, as determined with *cholineless*. Where the results are expressed on both a dry and a wet weight basis, the samples were first dried to con-

TABLE V

Choline Content of Some Natural Products

Material	Choline content per 100 mg.		Remarks
	Wet weight	Dry weight	
	γ	γ	
Bacon fat...........................	6.5		
" lean.......................	97		
Beet...............................	7.9	47	Fresh; root only
Butter............................	7.9		
Carrot............................	6.7	45	Fresh; root only
Coconut milk......................	0.03		
Yellow corn-meal..................		34	
Corn steep water concentrate.......	254	381	
" germ......................		67	
Egg white.........................	0.3	2.2	Hard boiled
" yolk.....................	1130	2170	" "
Dried whole milk..................		81	Commercial brand
Neurospora.......................	64	243	Wild type
Potato............................	19.5	67	New potato; skin included
Polished rice......................		89	
Rye flour.........................		104	
White wheat flour.................		119	
Dried brewers' yeast..............		262	Commercial brand

stant weight at 90°. In all other cases regular commercial products were used without further drying.

Genetic Analysis

Cholineless was crossed with the sex-linked character *albino*. The eight ascospores from each of twenty-one of the resulting asci were isolated in order and germinated on basal medium supplemented with choline. The cultures were then transferred to unsupplemented basal medium. Of each set of eight ascospores, four failed to grow in the absence of choline, showing that the mutation involved a single gene. In eleven sets the *cholineless*

gene segregated in the first division, while ten sets showed second division segregation. This corresponds to a map distance of approximately 25 units from the centromere. The gene is not linked with sex or with *albino*.

This work was supported by a grant from the Rockefeller Foundation. The original mutant was found and identified by Misses Helen Berman and Caryl L. Parker. A sample of pure lecithin was kindly provided by Professor J. W. McBain.

SUMMARY

A microbiological method for the determination of choline, by use of an artificially produced mutant of *Neurospora crassa*, has been described. Of more than forty compounds tested, only choline, lecithin, and methionine were found to support growth of the mutant. A rapid procedure for the separation of choline from methionine in tissue extracts is given. The *Neurospora* method is simple, sensitive, and specific.

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THE SPECIFICITY OF $l(-)$-METHIONINE IN CREATINE SYNTHESIS

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It has been demonstrated that methionine may act as the source of methyl groups for the physiological synthesis of creatine (1, 2). In the present study the specificity of l-methionine for this process has been examined by means of the *in vitro* technique of Borsook and Dubnoff (1).

EXPERIMENTAL

Creatine was estimated by the procedure of Borsook and Dubnoff (1), modified so that the final color could be conveniently measured in the Evelyn photoelectric colorimeter. In our earliest experiments the creatine measurements were accomplished by incubation with the Dubos and Miller organism, *Corynebacterium creatinovorans*[1] (3), and it was again established that the chromogenic material synthesized by liver slices under these conditions was truly creatine.

$d(+)$-Methionine ($\alpha_D = +7.1°$) was obtained from Dr. Madelyn Womack, $l(-)$-methionine from E. A. Staley and Company, Decatur, Illinois, dl-methionine from Merck and Company, Inc., and guanidoacetic acid from Hoffman-La Roche, Inc. Sodium α-keto-γ-methiolbutyrate (S calculated 18.84; found 18.79) was obtained from Mr. Guilford G. Rudolph, and dl-methionine sulfoxide (mol. wt. calculated 165.2; found 165.1), dl-methionine sulfone (mol. wt. calculated 182.4; found 181.2), and dl-methionine methylsulfonium iodide (I calculated 45.81; found 45.67) from Dr. Gerrit Toennies.[2]

A solution of dl-methionine methylsulfonium chloride was prepared by shaking 2 mM of the iodide in 1 cc. of water with 4 cc. of 0.25 M $HgCl_2$ for 1 hour. After centrifugation H_2S was bubbled through the supernatant fluid for 15 minutes, followed by CO_2 for 10 minutes. The mixture was filtered twice and the solution was then properly diluted for use in the incubation experiments. Complete protocols are not included herein, since they resemble so closely those of Borsook and Dubnoff.

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[1] This name, while not yet official, has been suggested to one of us (P. H.) by Dr. Dubos in a personal communication.

Adult rats of the Vanderbilt, Wistar, and Sprague-Dawley strains were used for each experiment. Considerable variation among individual rats was encountered. For the present purpose it was advantageous to use livers with which one could elicit a large response in the presence of guanido-acetic acid and methionine and a considerably smaller one in the presence of guanidoacetic acid alone. However, not infrequently the effect of methionine addition was only 10 to 20 per cent of that due to guanidoacetic acid and in such instances the results of the experiment were discounted. Except in the very first trials guanidoacetic acid was employed at a concentration of 0.1 mg. per vessel and methionine and its derivatives at 1.25 mg. Both concentrations were considerably lower than those used by Borsook and Dubnoff and were found to be equally effective.

Effect of d-Methionine and of α-Keto-γ-methiolbutyric Acid—The results are summarized in Table I. $Q_{creatine}$ is the amount of creatine formed expressed as if it were a gas in c.mm. at s.t.p. per mg. of tissue (dry weight) per hour. Each figure is the mean value determined in six experimental trials, each in triplicate. ΔQ is the increment in $Q_{creatine}$ over that determined

TABLE I
Effect of d(+)-Methionine in Creatine Synthesis

Substrate	$Q_{creatine}$	ΔQ	$Q_{creatine}$	ΔQ
Guanidoacetic acid........................	1.98		2.02	
" " + l-methionine.........	3.90	1.92	3.95	1.93
" " + d-methionine.........	3.00	1.02		
" " + α-keto acid.........			3.91	1.89

with glycocyamine alone. The values presented in the first pair of columns, comparing d- and l-methionine, were obtained with the same set of six livers, while those in the second pair of columns were obtained with a second series of six livers. It will be seen that $d(+)$-methionine appeared to be about 50 per cent as potent as its optical antipode for creatine synthesis. This was quite consistent in each experiment. The α-keto analogue of methionine appeared to be fully as active as the parent substance.

From the data it was not apparent whether $d(+)$-methionine and the α-keto acid were themselves capable of methylating guanidoacetic acid or whether they were first converted to l-methionine by the liver tissue. The first step in such a transformation would be, presumably, the oxidative deamination of the d-amino acid. It has been found that benzoic acid inhibits the activity of the d-amino acid oxidase (4). In Table II are presented the results of a group of six experiments, each performed in triplicate, in which the effect of benzoic acid on the ability of the liver slices to utilize d-methionine for creatine synthesis was studied. It will be seen that 0.01 M benzoic acid completely prevented creatine synthesis by d-methionine but

in no wise impaired creatine synthesis by the natural isomer. It appears, therefore, that d-methionine cannot, itself, serve as a creatine precursor but must first be oxidatively deaminated. Since no substance has been reported to inhibit the amination of α-keto acids by tissue slices, we could not determine whether the α-keto acid can directly serve as a methyl donor for guanidoacetic acid or whether it must first be resynthesized to l-methionine.

Effect of Toennies' Compounds in Creatine Synthesis—It has been shown by Bennett that while dl-methionine sulfoxide (5) and methylsulfonium iodide (6) can replace methionine in the diet of the white rat, dl-methionine sulfone (6) cannot do so. It was thought of interest to correlate these

TABLE II

Effect of Benzoic Acid on Creatine Synthesis

Substrate					$Q_{creatine}$	ΔQ
Guanidoacetic acid					2.75	
"	"	+ benzoate			2.53	−0.22
"	"	+ l-methionine			5.05	2.30
"	"	+	"	+ benzoate	4.96	2.21
"	"	+ d-methionine			4.45	1.70
"	"	+	"	+ benzoate	2.68	−0.07

TABLE III

Oxidation Products of Methionine in Creatine Synthesis

Substrate				$Q_{creatine}$	ΔQ
Guanidoacetic acid				3.20	
"	"	+ l-methionine		4.89	1.69
"	"	+ dl-methionine sulfone		3.16	−0.04
"	"	+	" sulfoxide	3.18	−0.02
"	"	+	" methylsulfonium chloride	4.70	1.50

facts with the ability of these substances to replace methionine for creatine synthesis. The results are presented in Table III. The figures in the table represent mean values for four determinations, each performed in triplicate. Each substance was used at the same molecular concentration employed for methionine.

Neither the sulfoxide nor the sulfone effectively enhanced the ability of liver slices to methylate guanidoacetic acid. Assuming that the liver cells are as freely permeable to the sulfoxide and sulfone as they are to methionine itself, it would appear that these oxidation products of methionine cannot be normal intermediates in the methylating process which functions *in vitro*.

The failure of methionine sulfoxide to accelerate creatine synthesis *in vitro* is difficult to interpret in view of the reported lipotropic activity of this substance (7). If the lipotropic activity of both methionine and its sulfoxide is determined only by the availability of their S-methyl groups for choline synthesis (8), and if the transmethylation mechanisms in choline and creatine synthesis are similar, then it must appear that while oxidation of the sulfur atom of methionine does not occur in transmethylation *in vitro* the possibility still remains that an independent transmethylation system, capable of utilizing methionine sulfoxide *per se*, does function in the intact animal. From the available evidence, however, it appears more likely that the intact animal possesses some mechanism whereby methionine sulfoxide may be reduced to the parent substance which may then be utilized for choline synthesis.

The methylsulfonium chloride appeared to be fully as active as methionine in creatine synthesis. However, the mechanism of this reaction is not understood. The present findings are compatible with the reported lipotropic activity of $d(+)$-methionine (9), since choline, like creatine, could be synthesized after the physiological conversion of d- to l-methionine.

SUMMARY

$d(+)$-Methionine is about 50 per cent as active as the natural isomer in creatine synthesis by liver slices *in vitro*. Benzoic acid, which inhibits the d-amino acid oxidase, also inhibits this reaction but does not interfere with transmethylation by $l(-)$-methionine. The sulfoxide and sulfone of dl-methionine are ineffective in creatine synthesis but the methylsulfonium chloride and the α-keto analogue of methionine are as active as methionine itself. Oxidation of the sulfur atom of methionine does not appear to occur in transmethylation.

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EXPERIMENTAL ALKAPTONURIA IN THE WHITE RAT ON HIGH TYROSINE DIETS*

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Excretion of homogentisic acid has been produced experimentally in rats by the feeding of large amounts of phenylalanine (1–3) and in ascorbic acid-deficient guinea pigs by phenylalanine or tyrosine administration (4). The experiences of various workers with regard to excretion of homogentisic acid in rats fed tyrosine have varied. Butts, Dunn, and Hallman (3) found no trace of homogentisic acid in the urine of rats fed *dl*-tyrosine, whereas the feeding of *dl*-phenylalanine produced an alkaptonuria. Later, Butts, Sinnhuber, and Dunn (5) noted "considerable quantities" of homogentisic acid excreted by rats receiving *l*-tyrosine. Closs and Braaten (6) also noted alkaptonuria in rats receiving *l*-tyrosine dissolved in an equivalent amount of aqueous sodium hydroxide. Martin and Hueper (7), however, state that homogentisic acid was not found in the urine of rats which in their experiments received diets containing 10 per cent *l*-tyrosine.

In the course of studies to determine whether homogentisic acid would be formed and excreted in experimentally induced alkaptonuria in amounts sufficient to give luminescence under conditions described previously (8), we have consistently obtained excretion of large amounts of homogentisic acid by rats fed the following high tyrosine diet: dried skim milk powder 45, corn-starch 15, butter 25, cod liver oil 3, *l*-tyrosine 12 per cent. A supplement of 0.9 gm. of powdered brewers' yeast was thoroughly mixed with the amount of food given each day. Commercially available *l*-tyrosine (Merck or S. M. A. Corporation) which gave theoretical values for nitrogen content on analysis was used.

Rats of either sex, weighing about 100 gm., were kept in individual cages which were set on glass funnels provided with a paraffined wire screen and a small plug of glass wool to prevent feces from falling into the urine. The urine was collected under toluene in a small bottle. At the end of each 24 hour period the urine was examined by the alkali test and estimations of the homogentisic acid content were made by the method of Briggs (9). The values for homogentisic acid obtained in this way in some typical experiments are presented in Table I.

The errors involved in the estimation of homogentisic acid in these urines may detract from the value of these data from a quantitative view-point,

TABLE I

Excretion of Homogentisic Acid by Rats on 12 Per Cent l-Tyrosine Diet

Rat No.	Sex	Weight	Days on diet	Food intake		Homogentisic acid excreted	
				Total	l-Tyrosine	mg. per cc.	mg. per day
		gm.		gm.	gm.		
3	M.	95	1	10.0	1.20	0.0	0.0
			2	7.9	0.95	0.0	0.0
			3	5.9	0.71	0.7	8.9
			4	10.0	1.20	15.2	38.0
			5	10.0	1.20	13.8	96.6
			6	10.9*	0.00	0.0	0.0
4	"	102	1	9.9	1.19	0.0	0.0
			2	6.9	0.83	0.6	4.0
			3	7.4	0.89	8.5	68.0
			4	9.9	1.19	12.1	102.9
			5	6.4	0.77	10.1	25.3
			6	6.4	0.77	5.1	30.6
			7	5.9	0.71	7.7	61.6
			8	8.9	1.07	10.0	90.0
			9	3.9	0.71	6.4	48.0
5	F.	103	1†	9.9	1.19	4.3	17.2
			2	10.0	1.20	9.8	68.6
			3	10.0	1.20		‡
			4	10.0	1.20	12.6	113.0
			5	10.0	1.20	13.9	111.2
			6	10.0	1.20	8.2	59.0
			7	9.4	1.13	9.2	121.3
			8	9.9	1.19	10.7	130.0
			9	10.0	1.20	10.9	76.3
14	M.	137	1†	8.9	1.07	4.5	94.1
			2	8.4	1.01	4.4	135.0
			3	8.4	1.01	4.3	100.0
15	"	130	1†	10.0	1.20	0.0	0.0
			2	10.0	1.20	16.3	49.0
			3	10.0	1.20	33.0	138.0
16	"	112	1†	10.0	1.20	3.2	6.4
			2	7.9	0.95	2.0	7.0
18	F.	96	1	8.6	1.03	0.0	0.0
			2	5.4	0.65	0.0	0.0
			3	6.5	0.78	0.8	17.7
			4	3.9	0.47	4.0	100.8

* Control diet, l-tyrosine replaced by starch.
† Fasted previous 24 hours.
‡ Urine lost through accident.

but such data indicate well the time of onset and the degree of intensity of the induced alkaptonuria.   In general it may be said that an intense alkaptonuria has been induced in all of the eighteen rats that we have had on this high tyrosine diet.   The excretion of homogentisic acid usually appeared on the 3rd day on the diet but could be made to appear as early as the 1st day if the rat were fasted for the previous 24 hours (cf. Rats 5, 14, 16). The diet induced an alkaptonuria in both sexes.   The excretion of homogentisic acid disappeared the 1st day after the rat was taken off the diet (cf. Rat 3, Day 6).   Rats kept on a control diet in which additional corn-starch (12 per cent) replaced the 12 per cent of l-tyrosine developed no alkaptonuria.

The excretion of homogentisic acid by these animals was confirmed by its actual isolation from the urine.   The concentration was great enough so that the acid could be precipitated directly from the urine as the lead salt, and homogentisic acid was recovered from the lead homogentisate by the method of Garrod (10).   Lead homogentisate was recovered regularly from these urines and a typical isolation is described.

The urine of four rats was collected for 4 days after the onset of alkaptonuria.   The combined urine (80 cc.) was brought to a boil and 5 gm. of lead acetate were added with stirring, and the mixture was filtered hot. The light brown solution was allowed to stand in the cold.   Crystallization began in about 2 hours and a nice crop of light tan crystals precipitated overnight.   This was filtered off with suction and, air-dried, weighed 0.754 gm.   This product was pure enough for most purposes.   Recrystallization once from water made slightly acid with acetic acid and treatment with a small amount of norit A gave pure white crystals of lead homogentisate which, air-dried, weighed 0.297 gm.   0.2968 gm. lost 0.0268 gm. on drying at 100° for $1\frac{1}{2}$ hours.   This represents a loss of water of crystallization of 9.03 per cent.   Theory for water of crystallization in lead homogentisate $(C_8H_7O_4)_2Pb \cdot 3H_2O$ is 9.08 per cent.   0.264 gm. of the anhydrous lead salt was suspended in ether and the lead removed with H_2S.   After filtration from the lead sulfide the ether was evaporated at room temperature, and the resulting pure white crystals after drying weighed 0.138 gm.   The acid melted at 148–149° (uncorrected) and did not depress the melting point of homogentisic acid prepared in a similar manner from human alkaptonuric urine.   Both the lead salt and the acid gave the usual reactions characteristic of homogentisic acid, including luminescence.

With this diet it was possible to produce experimentally urines exhibiting the phenomenon of chemiluminescence previously noted in the urine of human alkaptonuric subjects which has been shown to be a property of homogentisic acid (8).   Strongly luminescent urines can usually be obtained in several days with rats on this diet.   In many cases the concen-

tration of homogentisic acid was so great that chemiluminescence was observed with 1:25 aqueous dilutions of the urine. Studies on known dilutions indicated that the minimum concentration of homogentisic acid necessary for visible luminescence in these urines was in the neighborhood of 0.6 mg. per cc. of urine. Similar experiments with pure homogentisic acid indicated a minimum concentration of about 0.55 to 0.60 mg. per cc. of homogentisic acid necessary for faintly visible chemiluminescence. In several cases rat urines containing high concentrations of homogentisic acid (25 to 30 mg. per cc.) would not show luminescence when undiluted but gave very strong luminescence in dilutions from 1:1 up to 1:25. Since pure homogentisic acid in a concentration of 30 mg. per cc. exhibited strong luminescence, it would seem that the appearance of light may be inhibited in the undiluted experimental urines when the excretion of tyrosine metabolites becomes extremely high. That the luminescence of homogentisic acid can be inhibited by other substances can be shown with ascorbic acid. Sealock *et al.* (11) mentioned the fact that human alkaptonuric urines containing extra ascorbic acid did not darken on standing; *i.e.*, the homogentisic acid was protected against oxidation by atmospheric oxygen. Likewise the addition of ascorbic acid inhibits the luminescence of homogentisic acid, if enough is added to prevent the darkening of the urine when shaken vigorously with air after the addition of alkali. The concentration of ascorbic acid must be considerably greater than the concentration of homogentisic acid to inhibit the blackening when shaken as described. In the presence of 5 moles of ascorbic acid, for example, 1 mole of homogentisic acid was not oxidized by air when it was made alkaline and shaken vigorously many times. Blackening did not occur and neither did luminescence. With lesser amounts the luminescence was of lesser degree and slower to appear. Inhibition of the chemiluminescence of homogentisic acid was also obtained in a similar manner with *d*-isoascorbic acid.

It has been mentioned previously that hydroquinone does not exhibit chemiluminescence when made alkaline and shaken with air (8). We have also prepared and examined gentisic acid (hydroquinonecarboxylic acid) and have been unable to detect chemiluminescence with this compound under conditions wherein homogentisic acid is strongly luminescent. Gentisic acid has many of the properties of homogentisic acid; *e.g.*, its solutions turn dark on addition of alkali, give a blue color with ferric chloride, reduce Benedict's solution, reduce ammoniacal or neutral silver nitrate at room temperature, reduce the homogentisic acid reagent of Briggs, and, when made alkaline, reduce photographic paper. Since gentisic acid does not exhibit chemiluminescence, this property of homogentisic acid is a more specific test for the presence of homogentisic acid than are the usual qualitative tests and is useful in conjunction with them. This is of especial

interest since gentisic acid has been stated to occur in the urines of individuals receiving large amounts of sodium salicylate, particularly in cases of acute rheumatism (12), and gentisic acid in the urines of these individuals may cause the urine to behave in some respects like an alkaptonuric urine. Chemiluminescence has been observed by us in the urine of three different alkaptonuric human subjects. The concentration of homogentisic acid in both human and experimental alkaptonuria thus appears to be sufficient to exhibit chemiluminescence under the proper conditions, particularly when the diluted as well as the undiluted urine is tested.

SUMMARY

An intense alkaptonuria was produced in several days in white rats on a 12 per cent *l*-tyrosine diet. The alkaptonuria was confirmed by isolation of homogentisic acid from the urine.

The urine exhibited chemiluminescence when made alkaline and shaken with air. In some instances chemiluminescence was obtained in urine dilutions as high as 1:25.

Gentisic acid (hydroquinonecarboxylic acid) has many of the chemical properties of homogentisic acid but exhibited no chemiluminescence under conditions in which homogentisic acid is strongly chemiluminescent.

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CARBOHYDRATE CHARACTERIZATION*

IV. IDENTIFICATION OF d-RIBOSE, l-FUCOSE, AND d-DIGITOXOSE AS BENZIMIDAZOLE DERIVATIVES

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In previous papers of this series the formation of the benzimidazole derivatives of carboxylic acids has been applied to the identification of seven aldo-monosaccharides (1) and of hexuronic and saccharic acids (2). The benzimidazole procedure has now been extended to include the biologically important sugars d-ribose, l-fucose (6-desoxy-L-galactose), and d-digitoxose (2,6-didesoxy-D-allose). Since this work was completed, Richtmeyer and Hudson (3) have reported the physical constants of the free base of d-ribo-benzimidazole, in a paper dealing with the relation of optical rotation to configuration in the aldo-benzimidazole series. Their constants and ours are in agreement.

In common with the majority of the aldo-monosaccharides, d-ribose and l-fucose are converted to the aldo-benzimidazoles by oxidation of the aldose to aldonic acid by potassium hypoiodite in methanol, liberation of the acid from the precipitated potassium or barium salt, and condensation with o-phenylenediamine in the presence of hydrochloric and phosphoric acids at 135°. However, d-digitoxose is not amenable to this treatment because of the solubility of the potassium and barium salts of d-digitoxonic acid in methanol, while condensation of the acid, prepared by other methods, with o-phenylenediamine gives poor yields of d-digitoxo-benzimidazole. The derivative is readily obtained in good yields by application of the oxidative condensation of d-digitoxose with o-phenylenediamine in the presence of cupric acetate and acetic acid (4).

d-Ribose—In the oxidation step of the benzimidazole procedure of Moore and Link (1) d-ribonic acid separates largely as the potassium salt. The d-ribo-benzimidazole, formed in the condensation with o-phenylenediamine, is isolated with some difficulty because it is quite soluble in water in the presence of inorganic salts. One can separate about 50 per cent yields of nearly pure d-ribo-benzimidazole which may be recrystallized from about

10 parts of water after the separation from inorganic salts.[1]   The physical constants of *d*-ribo-benzimidazole and its secondary derivatives are given in Table I.

During the oxidation of *d*-ribose with alkaline hypoiodite in methanol, epimerization of the *d*-ribonic acid to *d*-arabonic acid occurs to the extent of about 5 per cent, the insoluble potassium *d*-arabonate precipitating with the potassium *d*-ribonate.   However, the *d*-arabo-benzimidazole, being very insoluble, is easily separated from the *d*-ribo-benzimidazole during the isolation.   This behavior of *d*-ribose (which may be shared by other sugars to a small or less easily detected extent) will interfere only in critical studies of mixtures in which the presence of small quantities of *d*-arabinose is being considered.   In such cases the bromine-barium benzoate procedure of Hudson and Isbell (5) may be used for the preparation of the *d*-ribonic acid, thus avoiding the epimerization reaction.   However, for general

TABLE I
Physical Constants of Aldo-benzimidazoles

The constants for the secondary derivatives are for the salts as isolated.   Melting points are uncorrected.

	M.p.	$[\alpha]_D^{25}$	Hydrochloride m.p.	Picrate m.p.
	°C.	degrees*	°C.	°C.
d-Ribo-benzimidazole	190	+22.5	196–198	185–186
l-Fuco-benzimidazole	248–249	−41.2	224–225	189–191†
d-Digitoxo-benzimidazole..............	207–209	−45.7	Oil	124–127

* Rotations in 1 N HCl with c = 2 (approximately).
† Sinters at 150°.

studies the bromine oxidation lacks the advantages of fractionation and convenience inherent in the methanol-hypoiodite method.

l-Fucose—The methanol-hypoiodite oxidation of *l*-fucose gives a precipitate only in the barium salt fraction, the yield being about 60 per cent.[2] Condensation of *l*-fuconic acid with *o*-phenylenediamine forms the very insoluble *l*-fuco-benzimidazole (similar in solubility to the stereochemically related *d*- or *l*-galacto-benzimidazole (3, 6)), whose constants are given in Table I.

[1] The modified scheme given in the experimental section for the isolation of the *d*-ribo-benzimidazole may be found useful for obtaining other rather soluble derivatives such as d-gluco-benzimidazole.

[2] A reason has not been established for the low yields of barium salt, or of benzimidazole in subsequent condensation, from several sugars which fall in the barium salt fraction.

d-Digitoxose—*d*-Digitoxo-benzimidazole is obtained in about 70 per cent yields by the oxidative condensation method (4). The constants of the derivative and its salts are given in Table I. Other sugars will interfere with the identification of *d*-digitoxose by this procedure only in so far as they may form very insoluble benzimidazoles (*e.g.*, *d*-galacto-benzimidazole) which would be difficult to separate from *d*-digitoxo-benzimidazole by recrystallization. Since the presence of 2-desoxy sugars is detected by characteristic qualitative reactions (7), the necessity for application of this special method of benzimidazole formation is easily ascertained.

EXPERIMENTAL

d-Ribose—Oxidation of 0.15 gm. (1 mM) of *d*-ribose by the hypoiodite-methanol procedure of Moore and Link (1) gives about 0.175 gm. (85 per cent) of K salt, m.p. 187° (decomposition), and 0.05 gm. of Ba salt.

Analysis—K salt. $C_5H_9O_6K$. Calculated, K 19.1; found, K 19.2–19.6. Ba salt, found, Ba about 41

The condensation is carried out according to the directions of Moore and Link (1); for each mM (0.204 gm.) of potassium salt, 0.14 gm. (1.3 mM) of *o*-phenylenediamine, 0.6 cc. (2.5 mM) of 4 N HCl, and 1.3 mM of H_3PO_4 are used. To the decolorized solution of the reaction mixture (volume 5 to 6 cc.) add 0.52 cc. of 5 N NaOH and 0.1 cc. of concentrated NH_4OH, cool, and extract three times with ether to remove unreacted *o*-phenylenediamine. Filter to remove any *d*-arabo-benzimidazole which may separate at this point. After removing the ether from the filtrate on the steam bath decolorize the warm solution and concentrate to dryness under reduced pressure. To the residue add 1 cc. of water with stirring at room temperature; place in the ice box for a couple of hours to permit solution of most of the inorganic salts and maximum crystallization of the *d*-ribo-benzimidazole. Filter the product and wash with a small amount of cold water, omitting the acetone wash since the derivative is appreciably soluble in the mixture of acetone and water which surrounds the crystals during the washing. Recrystallize the crude product (approximately 0.2 gm. containing some inorganic salts) from about 10 parts of water with decolorization. Any *d*-arabo-benzimidazole still present will be insoluble in this quantity of hot water and may be removed by filtration before decolorization and crystallization of the *d*-ribo-benzimidazole. The product thus obtained (about 0.10 gm. or 40 per cent of theory) has a melting point of 185–187°. An additional 10 to 15 per cent may be obtained by application of the copper salt precipitation (1) to the mother liquor from the crude *d*-ribo-benzimidazole and from the recrystallization. The constants for the purified *d*-ribo-benzimidazole ($C_{11}H_{14}O_4N_2$) and the hydrochloride

($C_{11}H_{15}O_4N_2Cl$) and picrate ($C_{17}H_{17}O_{11}N_5$), prepared according to the directions of Moore and Link (1), are given in Table I, with the analytical data in Table II.

The isolated *d*-arabo-benzimidazole (1), m.p. 235–237°, $[\alpha]_D = -45°$ in 1 N HCl, and picrate m.p. 155–156°, corresponds to between 1 and 5 per cent of the potassium salt in most cases. That the *d*-arabonic acid arises in the oxidation step by epimerization in the presence of the excess alkali was shown by preparation of *d*-ribonic acid from the same sample of *d*-ribose by the bromine-barium benzoate procedure of Hudson and Isbell (5), the condensation mixture with *o*-phenylenediamine giving no *d*-arabo-benzimidazole.

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TABLE II

Analyses of Ribo, Fuco, and Digitoxo Derivatives

</div>

The values are given in per cent.

	Carbon		Hydrogen		Nitrogen		Chlorine	
	Theory	Found	Theory	Found	Theory	Found	Theory	Found
d-Ribo-benzimidazole...... .	55.45	55.25	5.92	5.80	11.76	11.65		
" hydro-								
chloride..................	48.09	47.90	5.50	5.40	10.20	10.16	12.91	12.70
d-Ribo-benzimidazole picrate	43.69	43.72	3.67	3.40	14.99	15.05		
l-Fuco-benzimidazole	57.13	57.00	6.39	6.42	11.11	11.02		
" hydro-								
chloride....,........... .	49.92	49.85	5.93	5.85	9.70	9.61	12.28	11.95
l-Fuco-benzimidazole picrate.	44.91	44.85	3.98	3.85	14.55	14.32		
d-Digitoxo-benzimidazole	61.00	60.90	6.83	6.75	11.86	11.74		
"								
picrate............. ...	46.45	46.37	4.12	4.05	15.05	14.88		

l-Fucose (6-Desoxy-L-galactose)—The oxidation of 0.164 gm. (1 mM) of *l*-fucose gives no K salt and 0.250 gm. of Ba salt (Ba found, 31.4 per cent). The yield of *l*-fuconic acid in the barium salt fraction appears to be about 60 per cent of theory.[2]

Condensation of the fuconic acid from the barium salt with *o*-phenylenediamine and isolation by the Moore-Link procedure (1) yield 0.136 gm. (54 per cent, based on the *l*-fucose used) of nearly white, very insoluble *l*-fuco-benzimidazole, m.p. 253–254°, which is most conveniently purified by reprecipitation from the decolorized hydrochloric acid solution. The constants for pure *l*-fuco-benzimidazole ($C_{12}H_{16}O_4N_2$) and its secondary derivatives are given in Table I and the analyses in Table II. The rotation must be determined in 1 N HCl, since the derivative is not soluble in citric acid to the extent of 2 per cent. The hydrochloride ($C_{12}H_{17}O_4N_2Cl$) is ob-

tained in good yields by the Moore-Link method (1). The picrate $(C_{18}H_{19}O_{11}N_5)$ is a less satisfactory secondary derivative, since it separates from its aqueous solution in a gelatinous hydrated form.

d-Digitoxose (*2,6-Desoxy-D-allose*)—To 0.074 gm. (0.5 mM) of *d*-digitoxose add 0.06 gm. of *o*-phenylenediamine, 0.2 gm. of $Cu(OAc)_2 \cdot H_2O$, 2 mM of acetic acid, and 4 cc. of water and keep the mixture at 53° for 14 hours. After introducing H_2S to precipitate the copper, filter the solution hot and concentrate nearly to dryness at reduced pressure to remove most of the excess acetic acid. To the hot diluted solution (volume 5 to 10 cc.) add an excess of NH_4OH and allow the mixture to cool and crystallize. The crude isolated *d*-digitoxo-benzimidazole, which shows some solubility when washed with acetone, weighs 0.088 gm. (75 per cent of theory), m.p. 202–204°. The constants for *d*-digitoxo-benzimidazole $(C_{12}H_{16}O_3N_2)$, purified by reprecipitation from acid solution and recrystallization from ethanol, are given in Table I and the analyses in Table II. The picrate $(C_{18}H_{19}O_{10}N_5)$ tends to separate from the hot aqueous solution as an oil but crystallizes readily if seed crystals are added.[3]

SUMMARY

Methods are given for the identification of *d*-ribose, *l*-fucose (6-desoxy-*L*-galactose), and *d*-digitoxose (2,6-didesoxy-*D*-allose) as the benzimidazole derivatives of the corresponding aldonic acids.

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[3] We have so far not been able to prepare a crystalline hydrochloride of *d*-digitoxo-benzimidazole. This is in contrast to all other benzimidazoles in the sugar group studied to date.

$= -29.2°$ (CHCl$_3$; c, 3.302). Ohle reported a melting point of 120° and $[\alpha]_D^{20} = -30.32°$ (CHCl$_3$; c, 3.258). The constants that we obtained for the quinoxaline prepared by the reaction of d-fructose and o-phenylene-diamine under the conditions given by Ohle are a melting point of 190–191° (decomposition) and $[\alpha]_D^{20} = -85.4°$ (4 N HCl; c, 2.090).

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THE EFFECT OF A LOW POTASSIUM DIET AND OF DESOXY-CORTICOSTERONE ACETATE ON THE CATION CONTENT OF RAT ERYTHROCYTES AND MUSCLE*

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On the basis of cation content mammalian erythrocytes may be divided into Na cells (carnivores) and K cells (rodents, Primates, etc.). Seemingly the two cations are functionally interchangeable.

Experimental evidence of a dynamic equilibrium between internal and environmental cation concentrations of erythrocytes has recently been accumulating by use of radioactive K for K cells *in vivo* (1–4) and by use of radioactive Na for Na cells *in vivo* (5, 6) and *in vitro* (6).

It has previously been shown, by purely chemical means, that the cation content of red blood cells could be altered *in vivo* by a number of experimental procedures. Kerr (7) found changes in the K content of dog erythrocytes resulting from hemorrhage, insulin, and injections of sodium oxalate. Others (8–10) produced altered cation contents in the red cells of cats and rabbits in response to diminution in plasma cation content, presumably an osmotic effect.

More recently Danowski (11) and Harris (12) have demonstrated cation exchanges in human red blood cells *in vitro* associated with and apparently dependent upon metabolic activity of the cells, and the latter (12) has shown that a reciprocal shift of K and Na may occur, although under certain conditions both ions may move in the same direction.

In view of these results it seemed of interest to discover whether one might in greater or less degree convert K cells to Na cells by diminishing the potassium stores of the body, as Heppel (13) had succeeded in doing for muscle cells (but not liver). The muscle studies served as an index of the degree of K depletion with which to compare the results on the red blood cells.

EXPERIMENTAL

Three groups of albino rats of both sexes were used as follows: Group I, used as controls, was maintained on the stock diet of chow under cage conditions otherwise identical with the experimental groups; Group II was maintained for 41 to 49 days on a low K diet (13) containing 0.046 per cent K; Group III was fed a similar low K diet for 20 to 36 days and received in

addition subcutaneous injections of desoxycorticosterone acetate[1] (DCA). This was administered daily in 2.5 mg. doses (dissolved in sesame oil) for 14 to 20 days. The latter additional procedure was adopted in the hope of speeding and increasing the negative K balance. No increase in the negative balance, however, was achieved in the given experimental period. At the time of sacrifice the rats ranged in weight from 140 to 365 gm., the range in weight being much the same for all three groups.

At the end of the diet period the rats were anesthetized with sodium pentobarbital and bled by decapitation into 50 cc. centrifuge tubes containing a few mg. of heparin. In most cases the blood from two and sometimes three rats was pooled, as shown in Table I, but the muscles of each rat were analyzed individually, the average results of the two or three analyses being tabulated. The blood was immediately centrifuged and the plasma removed. A hematocrit of the packed cells revealed the amount of plasma remaining among them, for which correction was made in estimating red blood cell electrolytes and water. Suitable aliquots of cells and plasma were transferred to Erlenmeyer flasks for chloride measurements. The remaining cells and 4 or 5 cc. of plasma were placed in separate crucibles for dry weight determinations and for ashing preliminary to Na and K analysis. In the subsequent calculations the density of the cells was assumed to be 1.085. A muscle mass was removed from each hind leg (4 or 5 gm.) and freed of gross fat and connective tissue. One was placed in a platinum crucible for ashing and Na and K analysis; the other was used for Cl estimation. Use was made of the Sunderman and Williams (14) method for Cl measurement, and that of Butler and Tuthill (15) for Na. Potassium was measured by the method of Shohl and Bennett as modified by Fenn *et al.* (16). The dry ashing was carried out at a temperature of $550°$.

Results

These are reproduced in Table I, and are expressed in milliequivalents per kilo of water in the case of plasma and red blood cells. For muscle the K and the excess or intracellular Na contents are expressed in similar units per kilo of intracellular water (total water minus chloride space), and Cl per kilo of fresh tissue. Figures for the chloride space are given in cc. per kilo of fresh muscle.

A comparison of the average results for Group I given in Table I with those for Group II reveals the effect of the low K diet on the distribution of water and the inorganic constituents measured. The results obtained on plasma and muscle are in agreement with those obtained by Heppel (13). The decrease in the muscle water is apparently mainly at the expense of the extracellular fluid compartment. The red blood cells have lost 15 milli-

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[1] Courtesy of Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey.

TABLE I
Electrolyte Distribution in Rats

No. of rats	Water			Per kilo water									Per kilo fresh
				Plasma			R.b.c.			Muscle			
	Per liter plasma	Per kilo muscle	Per kilo r.b.c.	K	Na	Cl	K	Na	Cl	K	Na	Cl	Cl space

Group I. Normal

	cc.	cc.	cc.	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	m.eq.	cc.
2	926		654	5.35	153	107	155	13.6	77.4				
2	930	757	669	4.90	154	107	148	14.9	83.1	164	6.52	12.8	113
2	925		658	5.64	162	107	148	13.2	74.7				
2	922		666	5.13	159	109	153	12.7	73.8				
2	930	749	659	5.20	152	109				161	2.04	12.8	111
3	932	763	670	5.49	154	109	152	12.8	74.9	155	4.28	12.6	109
3	933	755	666	4.85	148	110	145	12.2	77.7	160	4.37	13.4	115
3	930	755	654	5.03	151	111	154	12.1	78.8	159	4.06	13.4	115
2	922	759	654	6.19	155	109	152	15.1	78.8	156	2.19	13.7	119
Average....	928	757	661	5.31	154	109	151	13.3	77.4	159	3.91	13.1	114

Group II. On low K diet

2	926	748	655	3.42	151	99.0	133	17.0	66.7	107	40.0	10.1	97
2	930	748	647	3.30	153	99.5	140	14.4	67.0	132	32.2	11.1	106
2	930	743	642	2.01	151	94.0	127	18.6	63.7	98	51.0	11.8	119
2	925	750	652	2.24	151	97.5	129	15.6	66.7	94	56.0	11.9	116
2	925	747	653	2.60	151	98.0	134	16.7	67.7	125	32.7	11.4	111
2	926	750	649	2.60	147	102.0	140	14.7	63.0	133	30.8	10.8	100
2	923	746	644	3.24	147	105.0	141	19.9	67.0	122	37.8	10.8	97
Average....	926	747	649	2.77	150	99.3	136	16.7	66.0	116	40.1	11.1	107
Difference..	−2	−10	−12	−2.54	−4	−9.7	−15	+3.4	−11.4	−43	+36.2	−2.0	−7

Group III. On low K diet, receiving injections of DCA

2	927	757	661	3.06	153	94.8	142	21.5	64.3	126	40.0	12.5	125
2	923	754	668	2.77	159	94.6	148	19.0	63.8	114	43.0	11.8	124
1	935	755	662	3.01	154	94.0	152	17.9	65.3	124	39.6	10.6	107
1	934	755	653	2.74	152	95.6	152	19.1	66.4	120	42.0	12.2	121
2	923	757	651	2.69	155	95.5	156	17.8	65.2	118	43.7	11.6	107
1	929	754	650	2.42	159	95.0	152	18.9	66.2	116	45.5	13.0	130
1	930	759	645	2.25	159	93.5	152	15.1	66.6	111	47.2	12.3	125
Average....	929	756	656	2.71	156	94.8	151	18.5	65.4	118	43.0	12.0	120
Difference..	+1	−1	−5	−2.60	+2	−14.2	0	+5.2	−12.0	−41	+39.1	−1.2	+6

equivalents of K of which only 3.4 (23 per cent) are replaced by Na, resulting in a cation decrease of 11.6 milliequivalents compared to a plasma decrease of 6.5 milliequivalents. The diminished red cell Cl parallels that in the plasma and exactly balances the diminished cation concentration.

When injections of DCA are used in conjunction with the low K diet, the differences in the results on muscle and plasma are those which are to be expected on the basis of the known physiological properties of DCA (17, 18).

The complete absence of any change in the red blood cell K compared to the normal was surprising in view of the results in Group II. It was at first thought to be due to the shorter time on the low K diet, but in view of the fact that the muscle and plasma K had dropped to the same level as in Group II this idea was abandoned.

The diminished plasma and red blood cell Cl concentrations unaccompanied by cation decreases are due specifically to the effect of DCA, and must result in a greatly increased alkali reserve, but actual measurements of HCO_3 seem not to have been made in connection with DCA studies.

DISCUSSION

These results seem to indicate an extremely limited capacity on the part of K cells to make use of Na in the presence of a progressively diminishing bodily store of K. It would seem further that the red blood cell K levels observed in the two series of experiments are the reflection not of the K depletion in the body, but rather of the extent to which the total concentration of plasma cations, i.e. Na + K, has been altered.

Thus in Group II one observes a decrease in the K concentration of the red blood cells paralleling the decrease in plasma cations. In Group III the red cell K concentration is constant, as is the plasma cation concentration. In previous studies (10) red cell K was reduced, associated with a reduction in plasma cation concentration and a *rise* in plasma K.

That the change in the concentration of plasma cations may be looked upon as a purely osmotic influence so far as the red blood cells are concerned seems reasonable (10, 19). To accept this is simply to say that the K level of K cells *in vivo* will vary with the osmotic pressure of the plasma, and become stabilized at the new level by the same forces which are normally operative; i.e., metabolic and others.

SUMMARY

As a result of balance studies on plasma and erythrocytes (K cells) of rats subjected to prolonged negative K balance it is concluded that:

1. The K content of such cells reflects the concentration of total plasma cations and is not directly influenced by the plasma K level.

2. Certain of the functions mediated by K in such erythrocytes (perhaps

metabolic in nature) are not transferable to Na although the membranes are permeable to Na.

3. From (1) and (2) it may likewise be concluded that the factors or machinery for concentrating K in K cells differs from those normally functioning in muscle.

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THE ACTIVATION OF URICASE BY CYSTEINE*

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In a recent study of uricase, Davidson (2) reports that a number of sulfhydryl compounds have an "activating" effect on the enzyme. The following note extends these observations, with particular respect to cysteine.

Purification of Uricase—Uricase was prepared by a modification of the methods of Davidson (1) and Holmberg (3). The starting material was an acetone powder of pig liver. All extractions were carried out in a Waring blendor. The preliminary extraction with buffer at pH 7.4 was found to effect little purification of the enzyme, and was abandoned. The enzyme was precipitated at pH 9 with half saturated ammonium sulfate, suspended in water, protein material was removed by heating to 55° for 5 minutes, and the enzyme reprecipitated at pH 7 with ammonium sulfate, as in Davidson's method. The precipitate was suspended in water, the excess sulfate precipitated with barium acetate, and further clarification of the solution effected with alumina Cγ. Dialysis of the solution against distilled water resulted in a highly active precipitate which was found on tests with the Tiselius electrophoresis apparatus to consist of two components—uricase, and a yellow protein of unknown nature. Extraction of the precipitate with 0.1 M phosphate buffer, pH 7.4, effected the removal of much of this yellow material. Slow addition of saturated ammonium sulfate to a concentrated solution of the enzyme in borate buffer (0.1 M) at pH 9 removed still more. The most active uricase preparations obtained by this method had about 400 times the activity of pig liver powder, which is comparable to the degree of purity obtained by Davidson. This procedure is much simpler than that of Davidson, and avoids the danger of inactivation of the enzyme by barium ions which, in the hands of the present author, made Holmberg's procedure highly unsatisfactory.

Effect of Cysteine on Oxidation of Uric Acid by Uricase—The experiments reported here were all performed in a Warburg manometric apparatus. Uric acid (as lithium urate) and cysteine were placed in the main portion of

the Warburg vessel with 1.5 cc. of 0.2 M borate buffer, pH 9, and enough water to make the total volume 3 cc. Uricase, suspended in 0.1 M borate buffer, pH 9, was added from the side arm after equilibration in the water bath at 38°. The gas phase was air.

The figures in Table I represent the average rate of oxygen uptake in the first 10 or 15 minutes after addition of the enzyme, during which time the rate was essentially constant. It is apparent from Table I that the greater

TABLE I

Oxygen Uptake in Mixtures of Uric Acid, Uricase, and Cysteine

Uric acid	Uricase	Cysteine	Rate of oxygen uptake
mg.	arbitrary units*	mg.	c.mm. per min.
2.5	5.0		4.7
2.5	5.0	0.5	9.6
2.5	5.0	2.3	9.1
2.5	5.0	5.0	11.2
2.5		5.0	0.5
0.7	2.5	5.0	6.5
1.6	2.5	5.0	6.7
2.5	2.5	5.0	6.4
	5.0	1.6	0.5
2.8	5.0	1.6	9.3
2.8	1.0		1.1
2.8	1.0	0.9†	4.0
	1.0	0.9†	0.6

* 1 unit of enzyme will result in an oxygen uptake of 1 c.mm. per minute in the presence of 2.8 mg. of uric acid.

† Cysteine added as glutathione.

TABLE II

Total Oxygen Consumed in Complete Oxidation of 10 Micromoles of Uric Acid by Uricase in Presence of Cysteine

Cysteine added, *micromoles*.........	0	2.5	5	10	20	30	40
Oxygen consumed, *micromoles*......	5.4	6.0	6.1	7.9	10.5	12.0	13.3

rate of oxygen uptake in the presence of cysteine is not due to autoxidation of cysteine or to direct oxidation of cysteine by the uricase preparation. The increase in the rate of oxygen uptake produced by cysteine is independent of the amount of cysteine added unless the amount is very small. It is also independent of the concentration of uric acid. Only the concentration of enzyme has any marked effect on the rate of oxygen uptake.

Studies were made of the total amount of oxygen consumed when the reaction is allowed to go to completion. The results of such studies are

given in Table II. Colorimetric determination of uric acid in the reaction vessels after termination of the experiment showed that the uric acid had completely disappeared in every case.

The results of the experiments cited in Table II are consistent with the hypothesis that oxidations of uric acid and of cysteine proceed simultaneously and independently in the reaction vessels.

$$2 \text{ uric acid} + O_2 + 2H_2O \rightarrow 2 \text{ allantoin} + 2CO_2$$

$$4 \text{ cysteine} + O_2 \rightarrow 2 \text{ cystine} + 2H_2O$$
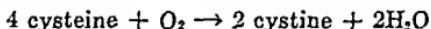
The excess oxygen used, beyond that which would be required for uric acid, is in each case approximately one-fourth the amount (in moles) of cysteine added. However, uricase alone, in the absence of uric acid, does not produce any increase in the slow autoxidation of cysteine, in itself insufficient to account for the observed increase in oxygen uptake.

SUMMARY

1. An improved method for the preparation of uricase is described.

2. It is shown that an increased uptake of oxygen follows the addition of cysteine to a mixture of uric acid with uricase.

3. The evidence presented indicates that this is due to a simultaneous oxidation of both cysteine and uric acid. Under the conditions of the experiments, however, cysteine is not oxidized rapidly by uricase in the absence of uric acid.

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STEROID EXCRETION IN A CASE OF ADRENOCORTICAL CARCINOMA

I. THE ISOLATION OF A Δ⁵-ANDROSTENETRIOL-3(β),16,17*

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Studies on steroid excretion in patients with the adrenogenital syndrome (1–6) have played an important rôle in the effort of identifying those urinary steroids that normally originate in the adrenal cortex. Moreoever, these investigations have led to the isolation of substances not yet encountered in other types of urine and thus have permitted further insight into the pathways of steroid metabolism in the body (7). As there appears to be a remarkable individuality among the few cases that hitherto have been reported, an extension of this work seemed clearly indicated. We were, therefore, most fortunate in having the cooperation of Dr. F. Harvie of the Department of Pediatrics of the University of Pennsylvania, who made it possible for us to collect urine from a 7 year-old boy with an adenocarcinoma of the adrenal cortex.

The object of the present communication is to report the isolation and chemical nature of a new steroid that has been obtained from this urine in considerable amounts. This substance is only sparingly soluble in most of the common organic solvents, a property which permitted the use of a very simple isolation procedure The urine was hydrolyzed with acid and extracted with ether. The neutral fraction of this extract was leached with benzene. The insoluble residue on repeated recrystallization yielded a product which showed a melting point of 270°. Identical material was obtained by hydrolysis of three analytically pure derivatives, two of which had been purified by the chromatographic method. The homogeneity of the parent substance was therefore considered to be established.

Analysis of this compound indicated the composition $C_{19}H_{30}O_3$. The 3 oxygen atoms are present as hydroxyl groups in reactive positions, as was

shown by the formation of a triacetate upon acetylation at room temperature. If this triol possessed a 4-ring carbon skeleton such as is present in the steroid molecule, the analytical data indicate the presence of one double bond. An ethylenic linkage could be demonstrated, as it was found that the substance decolorized 1 mole of bromine and that it could be hydrogenated catalytically to a triol of the composition $C_{19}H_{32}O_3$. The original compound was attacked by periodic acid. Although we failed to secure a primary reaction product in pure form, this instability towards periodic acid suggested nevertheless that at least two of the hydroxyl groups are located at adjacent carbon atoms. The results obtained in the oxidation of the saturated triol with chromic acid could readily be reconciled with this interpretation. Although this reaction was carried out at room temperature, no neutral products were obtained. The acidic fraction yielded a substance which had retained the original number of carbon atoms, as its analytical values were in close agreement with those of the formula $C_{19}H_{28}O_5$. The most plausible way to account for the changes in composition, the loss of 4 hydrogen atoms and the uptake of 2 oxygen atoms, is to assume that the reaction product is a ketodicarboxylic acid formed from the saturated triol by the opening of a ring. The acid $C_{19}H_{28}O_5$ upon treatment with m-dinitrobenzene and alkali developed an intense purple color. Among steroids such a behavior has been observed so far only with those substances that possess a carbonyl group at either C-3 or C-17 (8-10). It has also been shown that 3-ketosteroids reach a peak in color development after 5 minutes, while 17-ketosteroids do so after 1 hour (9). The differences in extinction are large enough to permit a ready differentiation between these two groups of compounds. Data presented in Fig. 1 demonstrate a satisfactory agreement between the absorption curves of the acid $C_{19}H_{28}O_5$ and of 3-ketoallocholanic acid when measured after reaction times of 5 and of 60 minutes. A markedly different curve would have been obtained with the unknown acid after 1 hour if its extinction at 520 mμ after 5 minutes had been caused by a keto group at C-17. This is illustrated by the behavior of androstenone-17 which showed a more than 3-fold increase in the height of its absorption maximum during the last 55 minutes of the reaction. It would appear from these observations that the unknown acid if derived from a steroid most likely possesses a carbonyl group at C-3.

If it is assumed that the original triol is indeed a steroid and has been converted into a 3-ketodicarboxylic acid, four possibilities exist for the location of the carboxyl groups. They may have been formed by ring cleavage between C-6 and C-7, C-11 and C-12, C-15 and C-16, or C-16 and C-17. The last possibility was considered the most probable one, as the isolated compound on distillation with potassium bisulfate (12) had yielded

a small amount of a product with the chromogenic properties of a 17-keto-steroid. Two acids with this structure (III), differing in the configuration at C-5, 3-ketoetioallobilianic acid and 3-ketoetiobilianic acid, have been described previously. Kuwada and Miyasaka (13, 14) prepared 3-keto-etioallobilianic acid from cholesterol and observed a melting point of 240° (corrected), whereas Marker and coworkers, who obtained the same acid from allopregnanetriol-3,16,20 (15) and from dihydrotigogenin (16), recorded melting points of 260° and 258°. 3-Ketoetiobilianic acid has been characterized by a melting point of 238° (17). As our product melted at 258°, it seemed possible that it was 3-ketoetioallobilianic acid. In order to
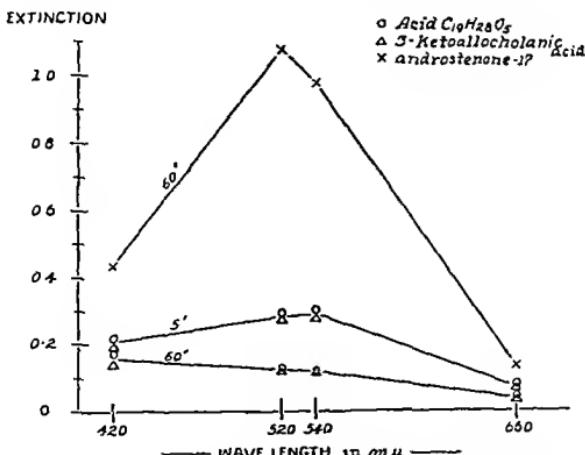
FIG. 1. Extinction ($-\log T$) of pigments formed with alkaline m-dinitrobenzene (Zimmermann reaction). Amounts tested: acid $C_{19}H_{28}O_5$ 120 γ (0.357×10^{-6} mole); 3-ketoallocholanic acid 134 γ (0.358×10^{-6} mole); androstenone-17 60 γ (0.220×10^{-6} mole; the extinction values obtained with androstenone (11) are plotted on such a scale that the extinction at λ 520 mμ after 5 minutes (0.16) would coincide with the extinction at λ 520 mμ for the acid $C_{19}H_{28}O_5$ after 5 minutes (0.28)). The measurements were carried out with an Evelyn photoelectric filter photometer; the reaction procedure was that described by Callow et al. (9).

make a direct comparison with an authentic specimen, isoandrosterone was converted into 3-ketoetioallobilianic acid. The procedure used (VI $\rightarrow$ VII $\rightarrow$ VIII $\rightarrow$ IX $\rightarrow$ III) was analogous to one employed by Marker and Wittle (18) in the degradation of pregnanol-3(α)-one-20 to 3-ketoetio-cholanic acid. It seemed suited for the purpose, as it had been shown by Stodola and Kendall (19) that 17-ketosteroids condense very readily with benzaldehyde. The 3-ketoetioallobilianic acid prepared in this manner proved to be identical with the oxidation product of the saturated triol. There was good agreement of the melting points and crystal forms of the free acids and their dimethyl esters and no depressions were observed on

mixed melting point determinations. The identity of these two substances proves conclusively that the triol $C_{19}H_{32}O_3$ is an androstanetriol-3,16,17 (II). The isolated compound (I) therefore is an androstenetriol-3,16,17. This leaves only the configurations of the hydroxyl groups and the location of the double bond undetermined.
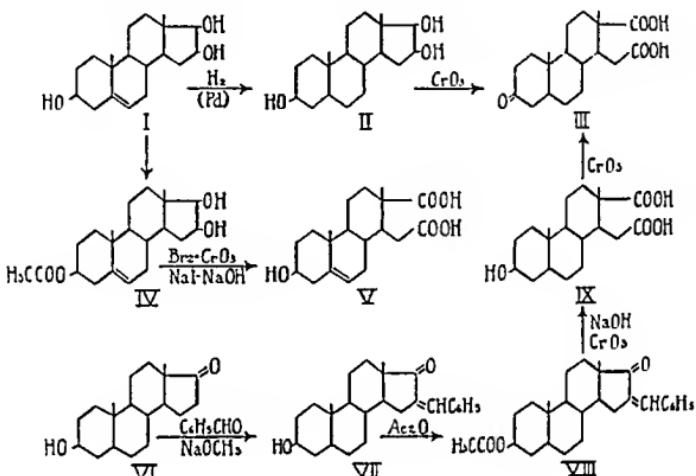
Androstanetriol and androstenetriol[1] yielded precipitates with digitonin when tested in a medium of 80 per cent ethanol. This indicated that these substances belong to the β series of 3-hydroxysteroids. Tentative evidence on the location of the double bond was adduced by a comparison of the optical rotations of androstenetriol triacetate and of androstanetriol triacetate. Upon reduction of the olefinic bond the molecular rotation increased by 25,000°. This suggested that the double bond is located between C-5 and C-6, as the reduction of no other isolated ethylenic bond in the steroid nucleus is known to produce an effect comparable to this in size and sign (21, 6). On this basis the isolated compound is to be formulated as a Δ^5-androstenetriol-3(β),16,17 (I). Definite proof for this structure was secured by the conversion of the 3-monoacetate of androstenetriol (IV) into β-3-hydroxy-Δ^5-etiobilienic acid (V). The starting compound for this reaction (along with the triacetate and a diacetate of androstenetriol) was obtained by chromatographic fractionation of the acetylated mother liquors of androstenetriol. The monoacetate was identified as a derivative of androstenetriol by hydrolysis and also by conversion of the resulting triol into androstenetriol triacetate. The presence of one acetoxyl group was ascertained by analysis, its location at C-3 by the course

[1] In the case of androstenetriol the reaction was quite incomplete, even when a saturated solution was used. The negative result previously reported (20) was obtained with a less concentrated solution.

of the oxidation with chromic acid. In this reaction bromine was used for the protection of the double bond and sodium iodide for the subsequent debromination (22). Acetylation of the acidic reaction product yielded the anhydride of β-3-acetoxy-Δ⁵-etiobilienic acid. β-3-Hydroxy-Δ⁵-etiobilienic acid was obtained by hydrolysis of the oxidation product and converted into the dimethyl ester and its acetate. The identities of the free acid and of its three derivatives were established by comparison with known compounds. A close agreement in melting points, solubilities, and crystal forms was observed, and no depression of the melting point occurred upon admixture of these reaction products with their reference specimens. The latter were obtained from dehydroisoandrosterone acetate. Ring cleavage was effected by oxidation with sodium hypoiodite, a procedure which recently has been described by Wettstein, Fritzsche, Hunziker, and Miescher (23).

The Δ⁵-androstenetriol-3(β),16,17 described in this paper is not identical with the Δ⁵-androstenetriol-3(β),16,17 which has been prepared from dehydroisoandrosterone by Butenandt, Schmidt-Thomé, and Weiss (24) and by Stodola, Kendall, and McKenzie (25). The difference between the two substances must be sought in a different spatial arrangement at C-16, or C-17, or both. A similar situation exists in the estrogen series, since estrone if subjected to Stodola's procedure does not yield estriol, the naturally occurring 16,17-glycol, but one of its stereoisomers (26). It must be emphasized, however, that the evidence at hand does not permit us to conclude that urinary estriol and our product possess the same configurations at C-16 and C-17. If such were the case the isolated androstenetriol would bear the same structural relationship to dehydroisoandrosterone as natural estriol does to estrone. This question seems of considerable interest as identity of the configurations of the glycol groups would lend support to any theory which would postulate that both estriol and androstenetriol are formed by the same reaction mechanism *in vivo*. It has been shown that the human body can convert estrone into estriol (27). The question arises therefore whether androstenetriol can be regarded as a metabolite of dehydroisoandrosterone. Since this 17-ketosteroid has been found in inordinately large amounts in several cases of adrenocortical carcinoma (1, 6), our isolation of very large amounts of the triol could readily be explained on the basis of such a theory. It is less easily understood, however, why androstenetriol has not yet been encountered in other cases of this type. Aside from dehydroisoandrosterone no other steroid has been isolated from natural sources which bears sufficient structural resemblance to androstenetriol to warrant its discussion as a possible precursor of the triol at this time. It is of interest, however, that the conversion of a 17-ketone into a 16,17-glycol does not seem to be the only mechanism whereby the

animal body can introduce a hydroxyl group into position 16 of the steroid nucleus.   At least it is difficult to visualize how a 17-ketosteroid could have acted as a precursor of allopregnanetriol-3(α),16,20 (15) which has been isolated from the urine of pregnant mares (28).

Δ^5-Androstenetriol-3(β),16,17 could not be extracted from urine with ether prior to acid hydrolysis.   It is believed therefore that the compound was excreted in conjugated form.   This seems to apply also to other steroids present in this urine, as the total neutral fraction and the neutral 17-keto-steroids which were extracted with ether from a non-hydrolyzed specimen comprised only 1 per cent of the amounts, respectively, that were liberated by hydrolysis.   In this regard our findings are in contrast to those of Butler and Marrian (2, 3) who obtained substantial amounts of free steroids from subjects with the adrenogenital syndrome.   A possible explanation for this difference can be found in the suggestion of Callow, Callow, Emmens, and Stroud (29) that the observations of Butler and Marrian (3) as well as similar findings of Broster *et al.* (30) did not reflect the original state of 17-ketosteroids and androgens in urine but were occasioned by its decomposition.

EXPERIMENTAL[2]

Collection and Fractionation of Urine—The clinical and pathological find-ings on C. K., a 7 year-old boy with an adenocarcinoma of the left adrenal cortex and extended metastases in liver and lungs, will be published else-where.   12.4 liters of urine were collected during the last 4 weeks of his life.   The urine was placed in an ice chest immediately after being voided and was worked up every other day.

11.4 liters of this urine were fractionated according to the following pro-cedure.   1 volume of urine was brought to a boil, acidified with 0.05 volume of concentrated hydrochloric acid, heated under a reflux for 20 minutes, and allowed to cool.   0.1 part of sodium chloride was added and the mixture extracted with 1 volume, 3 times with $\frac{1}{2}$ volume of ether, and twice with $\frac{1}{2}$ volume of ethyl acetate.   The ether extracts were combined, washed with a small amount of water, extracted with 1 volume and twice with 0.25 vol-ume of 1 N sodium hydroxide solution (per 1 volume of urine), washed until neutral, and taken to dryness.   These steps were taken in rapid succession, as otherwise an almost colorless precipitate would form in the ether phase.[3]   The weights of the neutral fractions of the ether extracts increased during the collection period from 1.26 to 3.56 gm. per liter of urine, 36.9 gm. being obtained altogether.

The alkaline extracts were acidified with concentrated hydrochloric acid

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[2] All melting points reported are corrected.
[3] Δ^5-Androstenetriol-3(β),16,17 has been isolated from such a precipitate.

and the salt concentration raised to 9 per cent. 1 volume of this mixture was extracted with 0.75 volume of ether in three portions. The ether extracts were washed with small amounts of water and taken to dryness. The residues derived from 11.3 liters of urine were taken up in 3 liters of ether and extracted five times with 5 per cent sodium carbonate solution (totaling 3.4 liters). The ether phase was washed with water and yielded 1.26 gm. (phenolic fraction). The sodium carbonate extracts were acidified, salted, and extracted with ether (3.4 liters) which yielded 9.19 gm. (acidic fraction).

The ethyl acetate extracts (derived from 8.9 liters of urine) were extracted with sodium carbonate and with sodium hydroxide solutions. The neutral fraction yielded 3.53 gm. of dark brown, partly crystalline material.

17-Ketosteroid Assays—The 17-ketosteroids in the neutral fractions of the ether extracts were determined according to the method of Callow *et al.* (9). They amounted to 320 mg. per liter (275 mg. per 24 hours) on the 28th day and to 740 mg. per liter (420 mg. per 24 (?) hours) on the last day before death and represented 25 and 24 per cent, respectively, of the weights of the neutral fractions. The specimen collected on the day before death (565 cc.) had been extracted with ether also prior to acid hydrolysis. The neutral fraction of this extract (18.7 mg.) contained 4.4 mg. of 17-ketosteroids (24 per cent).

Isolation of Δ^5-Androstenetriol-3(β),16,17—45 cc. of benzene[4] were added to the neutral fraction (12.99 gm.) of the ether extract of 4.20 liters of urine (collected on 8 days in the period from the 19th to 10th day before death). The solvent was brought to a boil and the mixture kept at room temperature for 20 hours. The insoluble material was washed with 21 cc. of benzene (in four portions) and weighed 1.54 gm. Upon five recrystallizations from ethanol and methanol 85 mg. of colorless hexagonal prisms were obtained, melting at 265–270° with decomposition. The fractionation of the mother liquors is described below.

Analysis—Sample recrystallized from ethanol and dried at 110° *in vacuo*
$C_{19}H_{30}O_3$. Calculated, C 74.47, H 9.87; found, C 74.54, H 9.87

Androstenetriol is readily soluble in pyridine and in glacial acetic acid. It is moderately soluble in ethanol, less so in methanol, and only sparingly soluble in acetone, ether, and benzene. A saturated solution of androstenetriol in 80 per cent alcohol was mixed with a solution containing an equivalent amount of digitonin (1 per cent in 80 per cent alcohol). A small amount of a flocculent precipitate formed within 1 hour. Androstenetriol when tested in the Liebermann reaction turned purple, then faded, became

[4] Some batches contained resinous material which did not dissolve in benzene. In these instances a minimal amount of methanol was added to the benzene.

green, and finally yellow-brown. The androgenic potency of androstenetriol has not yet been determined.

Δ^5-*Androstenetriol-3(β),16,17 Triacetate*—1 cc. of acetic anhydride was added to a solution of 33.9 mg. of androstenetriol in 2 cc. of pyridine. After the mixture had stood at room temperature for 15 hours, the excess reagent was hydrolyzed by the gradual addition of 1 cc. of cold water. The solution was distributed between benzene and water. The benzene layer was washed repeatedly with dilute hydrochloric acid and with water and was taken to dryness. The residue (47.2 mg.) was recrystallized from 95 per cent alcohol and yielded 37 mg. of hexagonal plates melting at 187–188.5°. After storage for 18 months the melting point of this preparation was 189.5–191°.

Analysis—Sample dried at 80° *in vacuo*
  $C_{25}H_{36}O_6$. Calculated, C 69.42, H 8.39; found, C 69.23, H 8.43
Rotation—$[\alpha]_D^{23} = -102°$ (1% in 95% ethanol)

21.2 mg. of androstenetriol triacetate (m.p. 188.5°), 21 mg. of sodium hydroxide, and 2.3 cc. of 90 per cent alcohol were heated under a reflux for 30 minutes. Ethyl acetate was added, and the mixture was washed three times with water and taken to dryness. The residue (14.3 mg.) was recrystallized twice. This preparation of androstenetriol melted at 267–270°.

Fractionation of Mother Liquors of Δ^5-*Androstenetriol-3(β),16,17*—The first two mother liquor fractions obtained in the purification of androstenetriol were extracted with benzene, dried, and acetylated at room temperature with acetic anhydride and pyridine. The third to fifth mother liquors were acetylated in the same manner. 266 mg. of androstenetriol triacetate melting at 185.5–187.5° were obtained by recrystallization. The remainder of the acetylated material (1044 mg.) was extracted with 55 cc. of petroleum ether containing 20 per cent of benzene. 17 mg. of an amorphous product remained undissolved and were discarded. The solution was passed through a column (150 mm. × 19 mm.) of Brockmann's aluminum oxide (manufactured by Merck and Company, Inc., Rahway, New Jersey). The chromatogram was developed with petroleum ether containing 20 per cent (995 cc.), 30 per cent (500 cc.), and 50 per cent (1680 cc.) of benzene, with benzene (1950 cc.), with benzene containing 25 per cent (1000 cc.) and 50 per cent (950 cc.) of ether, with ether (845 cc.), with ether containing 25 per cent (1340 cc.) and 50 per cent (1250 cc.) of acetone, with acetone (1615 cc.), and finally with methanol (1470 cc.). This was followed by extraction of the alumina with methanol in a Soxhlet apparatus. In this manner forty fractions were collected. Their examination has not yet been completed and a detailed account of this experiment will therefore not be given at this time.

The first eluate (12 mg.) was collected with petroleum ether containing 50 per cent of benzene (250 cc.). Fraction 2 (64 mg.) which was obtained with 250 cc. of the same solvent mixture was repeatedly recrystallized from methanol. 31 mg. of hexagonal plates were obtained melting at 186.5–188.5°. The melting point was not depressed by admixture with androstenetriol triacetate. The isolation of androstenetriol diacetate from Fractions 17 and 18 (the final eluates with benzene containing 25 per cent of ether (2 × 300 cc.)) and of androstenetriol monoacetate-3 from Fractions 27 to 33 (the eluates with the final 330 cc. of ether containing 25 per cent of acetone, with ether containing 50 per cent of acetone, and with the initial 600 cc. of acetone) will be described below. With the inclusion of these compounds a total of 401 mg. of Δ^5-androstenetriol-3(β),16,17 has so far been isolated in free form or as derivatives from these 4.2 liters of urine (95 mg. per liter). It is to be expected that additional amounts will be obtained from fractions not yet investigated.

Δ^5-*Androstenetriol-3(β),16,17 Diacetate*—Fraction 17 obtained in the chromatographic separation of the acetylated mother liquors of androstenetriol (92 mg.) was repeatedly recrystallized from dilute ethanol and from methanol. 48 mg. of rectangular plates were obtained with none, one, two, or rarely three or four of its corners cut by a facet. These crystals melted at 183–187°. The melting point was depressed (153–164°) by admixture with androstenetriol triacetate. Fraction 18 (38 mg.) yielded an additional 24 mg. of the same compound.

Analysis—Sample dried at 80° *in vacuo*
　　　$C_{23}H_{34}O_5$. Calculated, C 70.74, H 8.78; found, C 70.60, H 8.82

35.4 mg. of androstenetriol diacetate were dissolved in 11 cc. of methanol and treated with 0.5 cc. of an aqueous solution (4 per cent) of sodium hydroxide. The mixture which was kept at room temperature for 24 hours deposited large hexagonal prisms. An additional crop was obtained by the addition of water. The crystals were repeatedly washed with water, dried (27.1 mg.), and recrystallized from 95 per cent ethanol and from 90 per cent methanol. The melting point (266–270°, with decomposition) had remained constant during the last two recrystallizations and was not depressed by admixture with androstenetriol. The final product appeared to retain 0.5 mole of methanol, even at rather high temperatures *in vacuo*. Sample A was dried at 110°, Sample B at 135°.

Analysis—$C_{19}H_{30}O_3$.　　　Calculated. C 74.47, H 9.87
　　$C_{19}H_{30}O_3 \cdot \frac{1}{2}CH_3OH$.　　　"　　" 72.63, " 10.00
　　　　　　　　　　Found (A). " 72.72, " 9.95
　　　　　　　　　　　" (B). " 72.97, " 10.35

To confirm the identity of this product the triol was reacetylated with acetic anhydride and pyridine at room temperature. The final product

showed no depression of its melting point (189–190°) upon admixture with androstenetriol triacetate.

Δ^5-*Androstenetriol-3(β),16,17 Monoacetate-3*—All eluates containing androstenetriol monoacetate (Fractions 27 to 33, 133 mg.) were contaminated with colorless material that rapidly darkened upon exposure to air. The three middle fractions (54 mg.) were recrystallized three times from methanol. 13.5 mg. of colorless rectangular plates melting at 243–245° were obtained. The melting point could not be raised by further recrystallization. The other eluates were similarly purified, and the mother liquors combined, distilled in a high vacuum, and recrystallized. 56 mg. of androstenetriol monoacetate were obtained altogether.

Analysis—Sample dried at 100° *in vacuo*
$C_{21}H_{32}O_4$. Calculated, C 72.38, H 9.26; found, C 72.06, H 9.10

20.4 mg. of androstenetriol monoacetate were hydrolyzed in dilute methanol with sodium hydroxide at room temperature. The reaction product (17.5 mg.) was recrystallized from methanol. Its melting point (268–271°, with decomposition) could not be raised by further recrystallizations from 95 per cent ethanol or from methanol and was not lowered by admixture with androstenetriol.

Analysis—Sample recrystallized from methanol and dried at 110° *in vacuo*
$C_{19}H_{30}O_3 \cdot \frac{1}{2}CH_3OH$. Calculated, C 72.63, H 10.00; found, C 72.78, H 10.06

7.4 mg. of the free triol were reacetylated in 0.6 cc. of pyridine with 0.3 cc. of acetic anhydride. The ester (10.5 mg.) was recrystallized three times from dilute methanol and then showed a melting point of 187.5–189.5° which was not depressed by admixture with androstenetriol triacetate.

Androstanetriol-3(β),16,17—A solution of 34 mg. of androstenetriol in 16 cc. of ethanol was shaken in an atmosphere of hydrogen in the presence of 515 mg. of a 1 per cent palladium-calcium carbonate catalyst (31). The reaction ceased after 7 minutes, when approximately 1 mole of hydrogen had been taken up. The catalyst was removed by filtration. The reaction product after several recrystallizations from methanol melted at 256–260°.

Analysis—Sample dried at 110° *in vacuo*
$C_{19}H_{32}O_3$. Calculated, C 73.98, H 10.46; found, C 73.92, H 10.38

Androstanetriol is appreciably more soluble in ethanol and methanol than androstenetriol. Androstanetriol, dissolved in 80 per cent ethanol, formed a precipitate with digitonin almost instantaneously.

Androstanetriol-3(β),16,17 Triacetate—A solution of 78 mg. of androstenetriol triacetate in 14 cc. of 95 per cent alcohol was hydrogenated in the presence of 500 mg. of 1 per cent palladium-calcium carbonate catalyst.

The reaction product was freed of a small amount of material insoluble in acetone (but soluble in methanol) and recrystallized from methanol, in which it was less soluble than the starting compound. The final product melted at 175.5–176.5°. One sample of this preparation was dried at 80° *in vacuo* (Sample A), another by sublimation in a high vacuum (Sample B). The sublimate also melted at 175.5–176.5°.

Analysis—$C_{25}H_{18}O_6$. Calculated. C 69.09, H 8.81
Found (A). " 68.44, " 8.91
" (B). " 68.99, " 8.27
Rotation— $[\alpha]_D^3 = -44°$ (Sample A, 0.45% in 95% ethanol)

Oxidation of Androstanetriol-3(β),16,17; 3-Ketoetioallobilianic Acid—A mixture of 20.8 mg. of androstanetriol (m.p. 252–258°) in 2 cc. of glacial acetic acid and of 22.2 mg. of chromium trioxide in 0.3 cc. of 90 per cent acetic acid was kept at room temperature for 21 hours. The excess of chromic acid was reduced with methanol and the solution taken to dryness under reduced pressure. The residue was distributed between ether and water. The ether phase was washed with water, extracted three times with 4 per cent sodium hydroxide solution, washed with water, and evaporated. The residue weighed 0.2 mg. The alkaline extracts were acidified and extracted with ether. The acidic fraction yielded 15.1 mg. of colorless crystals. These were recrystallized from 50 per cent alcohol. The final product (7.7 mg.) melted at 253–256° with evolution of gas and formation of a brown pigment. A mixture with a specimen of 3-ketoetioallobilianic acid (m.p. 252–256°) prepared from isoandrosterone melted at 254–257°. Higher melting points (258°) were observed for both preparations of this acid when the samples were heated at a somewhat faster rate.

Analysis—Sample dried at 110° *in vacuo*
$C_{19}H_{28}O_5$. Calculated, C 67.83, H 8.39; found, C 67.72, H 8.30

2.8 mg. of 3-ketoetioallobilianic acid were dissolved in an ethereal solution of diazomethane. The resulting ester was recrystallized from 80 per cent methanol and melted at 131.5–134.5°. Admixture of an authentic specimen of 3-ketoetioallobilianic acid dimethyl ester (m.p. 135–136°) did not depress the melting point. The melting point recorded for this compound in the literature is 135° (15).

Oxidation of Δ⁵-Androstenetriol-3(β),16,17 Monoacetate-3; β-3-Hydroxy-Δ⁵-etiobilienic Acid—14.2 mg. of androstenetriol monoacetate (m.p. 239–242°) were dissolved in 1.2 cc. of glacial acetic acid. A solution of bromine in glacial acetic acid (about 68 mg. per cc.) was added until a faint yellow tint persisted for 15 minutes (0.13 cc.). The mixture was treated with a solution of 12.3 mg. of chromium trioxide in 0.2 cc. of 90 per cent acetic acid for 24 hours at room temperature, reduced with methanol, and evapo-

rated under reduced pressure. The residue was distributed between ether and 0.1 N hydrochloric acid, and the ether layer was washed repeatedly with water and taken to dryness. A solution of 25 mg. of sodium iodide in 5 cc. of acetone was added to this product. Iodine was formed immediately. The mixture was refluxed for 100 minutes, concentrated to a small volume, taken up in 60 cc. of ether and 10 cc. of water containing 0.2 per cent of sodium sulfite, and then shaken until colorless. The aqueous phase which was not separated was acidified and again extracted with the ether layer and then three more times with fresh ether. The ether solutions were combined, washed with water, extracted three times with a 4 per cent solution of sodium hydroxide, again washed with water, and taken to dryness (2.2 mg.). The alkaline extracts were acidified and extracted with ether, which yielded 10.5 mg. of acidic products. These were treated with 0.5 cc. of acetic anhydride and 1 cc. of pyridine at room temperature for 17 hours. Ether was added and the solution washed with water, dilute hydrochloric acid, sodium carbonate solution, and water. The acetylated product was recrystallized from methanol. 4.0 mg. of β-3-acetoxy-Δ⁵-etiobilienic anhydride were obtained. The melting point of this product (186–188°) was not lowered by admixture with a specimen of the anhydride (m.p. 186–188°) that had been prepared from dehydroisoandrosterone acetate (23).

Analysis—C$_{21}$H$_{28}$O$_5$. Calculated, C 69.98, H 7.83; found, C 70.50, H 8.11

The following melting points have been reported for this anhydride: 188° corrected (13, 14), 186° uncorrected (24), and 190.5–191.5° corrected (23).

The acidic fraction obtained in the oxidation of another batch of androstenetriol monoacetate-3 (14.4 mg.) was kept in alkaline solution (4 per cent sodium hydroxide) at room temperature for 17 hours before it was acidified and extracted with ether. The reaction product (7 mg.) was recrystallized from 65 per cent ethanol. 3.6 mg. of needle-shaped crystals were obtained that melted at 232–236°. Further recrystallization from dilute alcohol yielded 3.4 mg. of platelets melting at 247–255°. These two products appear to be allotropic modifications, as similar crystals and melting points were observed with an authentic preparation of β-3-hydroxy-Δ⁵-etiobilienic acid. (In this case a product melting at 250° yielded on recrystallization first needles melting at 236–237° and then platelets melting at 251–255°.) A mixture of the high melting form derived from androstenetriol with the low melting form of the reference compound melted at 247–255°. In all cases melting was accompanied by formation of a brown pigment and evolution of gas. β-3-Hydroxy-Δ⁵-etiobilienic acid has been reported to melt at 251°, corrected (13).

2.9 mg. of β-3-hydroxy-Δ⁵-etiobilienic acid were methylated with diazo-

methane. The ester was recrystallized from dilute acetone and from petroleum ether. Needles melting at 107.5–110° were obtained. The melting point of a mixture with β-3-hydroxy-Δ^5-etiobilienic acid dimethyl ester (m.p. 112–113°) prepared from dehydroisoandrosterone acetate was 109–113°. Kuwada (13) has reported a melting point of 112°, corrected, for this ester.

The reaction product (β-3-hydroxy-Δ^5-etiobilienic acid dimethyl ester) was combined with its mother liquor fractions and the methylated mother liquors of the free acid, and acetylated with acetic anhydride and pyridine at room temperature. The acetate was recrystallized from dilute methanol and from petroleum ether. The final product melted at 147–151.5°, and in mixture with an authentic sample of β-3-acetoxy-Δ^5-etiobilienic acid dimethyl ester (m.p. 152–153.5°) at 150.5–153°. The melting points (corrected) reported in the literature for this compound are 153° (13) and 156–157° (23).

Oxidation of Δ^5-Androstenetriol-3(β),16,17 with Periodic Acid—The use of aqueous ethanol as a reaction medium led to the formation of a yellow oil which could not be induced to crystallize. In the subsequent experiments a suggestion of Miescher, Hunziker, and Wettstein (32) was followed, who recommended the use of aqueous dioxane. A solution of 14 mg. of periodic acid ($HIO_4 \cdot 2H_2O$, supplied by the G. Frederick Smith Chemical Company, Columbus, Ohio) in 0.1 cc. of water was added to a suspension of 10.2 mg. of androstenetriol in 2.7 cc. of dioxane (purified according to Eigenberger (33)). The reaction vessel was filled with nitrogen, agitated occasionally until the triol had dissolved, and allowed to stand at room temperature for 16 hours altogether. During that time water-soluble, needle-shaped crystals had separated from the colorless solution. (These can be dissolved by reducing the dioxane concentration to 80 per cent. When this was done in another experiment, gradual liberation of iodine was observed and only a small yield of water-insoluble material was obtained.) The reaction mixture was distributed between 55 cc. of benzene and 12 cc. of water. A crystalline precipitate formed. This was separated by centrifuging and washed repeatedly with water. (Yield, 6.9 mg.) The product reduced Tollens' reagent promptly in the cold. It crystallized from dilute methanol or ethanol in rhombic plates and from acetone in fine needles and melted at 131–134°. The preparation used for analysis which was obtained from this material and from the residue of the washed benzene extract (2.7 mg.) melted at 132–133°. It was dried at 80° *in vacuo*. The analytical figures (found, C 70.08, H 8.95) do not agree at all with those calculated for Δ^5-androstenol-3(β)-dial-16||17 (C 74.96, H 9.27) which was expected to result from this reaction. The product used for analysis gave a negative Beilstein test for

halogens.   Attempts to obtain the dialdehyde were discontinued when the structure of the starting compound became established, as it seemed advisable to conserve the remainder of the material for the more urgent task of studying the configurations of the hydroxyl groups at C-16 and C-17.

Preparation of 3-Ketoetioallobilianic Acid

16-Benzylidenandrostanol-3(β)-one-17—A solution of 152.7 mg. of isoandrosterone in 5 cc. of methanol was mixed with a solution of sodium methylate (48.5 mg. of sodium in 1 cc. of methanol) and heated under a reflux for 85 minutes.   During the first 25 minutes 0.54 cc. of a mixture of benzaldehyde and methanol (1:4) was added in four portions.   Crystals formed on cooling.   The reaction mixture was distributed between ether and water, and the ether layer was washed twice more with water and taken to dryness.   The residue was recrystallized once from acetone. 176.2 mg. of hair-like needles were obtained which melted at 176.5–181.5°. This product was used for the next step.   A sample which was purified further for analysis melted at 181.5–182.5°.   It was dried at 80° *in vacuo.*

Analysis—$C_{26}H_{34}O_2$.   Calculated, C 82.49, H 9.05; found, C 82.10, H 9.42

16-Benzylidenandrostanol-3(β)-one-17 Acetate—166.9 mg. of benzylidenisoandrosterone were acetylated with 4 cc. of pyridine and 2 cc. of acetic anhydride at room temperature.   The crude acetate (175 mg.) was oxidized without purification.   Another preparation was recrystallized from methanol, in which it is only moderately soluble.   Hexagonal plates melting at 237–238° were obtained.

Analysis—Sample dried at 80° *in vacuo*
$C_{28}H_{36}O_3$.   Calculated, C 79.96, H 8.63; found, C 79.79, H 8.77

β-3-Hydroxyetioallobilianic Acid—A solution of 175 mg. of benzylidenisoandrosterone acetate in 24 cc. of glacial acetic acid was stirred and maintained at 60° for 5 hours.   220 mg. of chromic acid in 3 cc. of 90 per cent acetic acid were added dropwise during the initial 75 minutes.   The excess of the oxidant was reduced with methanol and the mixture taken to dryness *in vacuo.*   The residue was distributed between ether and water; the ether phase was washed with dilute hydrochloric acid and extracted with 1 N sodium hydroxide solution.   The ether yielded 7 mg. of a colorless oil. The alkaline extracts were kept at room temperature for 18 hours and then boiled for a few minutes.   An ether extract of the acidified solution yielded 156 mg. of a crystalline residue.   This was recrystallized from acetone and then repeatedly from 75 per cent alcohol.   β-3-Hydroxyetioallobilianic acid crystallized from this solvent in rectangular prisms which disintegrated on drying.   The top fraction (56 mg.) melted at 254–257° with evolution

of gas. The melt was colorless. The mother liquors were freed of benzoic acid by extraction with petroleum ether and yielded on recrystallization an additional 22 mg., melting at 249–253°. With the inclusion of this material the over-all yield of β-3-hydroxyetioallobilianic acid from isoandrosterone was 46 per cent.

$Analysis$—Sample dried at 110° $in\ vacuo$
$\qquad$ $C_{19}H_{30}O_5$. Calculated, C 67.43, H 8.94; found, C 67.59, H 8.96

The melting points of this acid varied somewhat with the rate of heating but were always distinctly higher than those recorded in the literature (239° corrected (14), 238° (34), 244–247° (16), and 246–247° (35)).

3-$Ketoetioallobilianic\ Acid$—40.8 mg. of β-3-hydroxyetioallobilianic acid were dissolved in 2 cc. of glacial acetic acid and treated with 12.5 mg. of chromic acid (in 0.17 cc. of 90 per cent acetic acid) at room temperature for 18 hours. The reaction mixture was worked up in the usual manner and yielded 38.8 mg. of colorless crystals. Upon recrystallization from 60 per cent alcohol 31.2 mg. of 3-ketoetioallobilianic acid were obtained in faceted prisms which melted at 253–257° with evolution of gas and formation of a brown pigment.

$Analysis$—$C_{19}H_{28}O_5$. Calculated, C 67.83, H 8.39; found, C 67.36, H 8.40

13 mg. of 3-ketoetioallobilianic acid were methylated with diazomethane. The dimethyl ester was recrystallized from dilute methanol and melted at 135–136°.

$Analysis$—Sample dried at 60° $in\ vacuo$
$\qquad$ $C_{21}H_{32}O_5$. Calculated, C 69.20, H 8.85; found, C 68.77, H 8.81

SUMMARY

A new compound of the composition $C_{19}H_{30}O_3$ melting at 267–270° has been isolated from the urine of a boy with an adrenocortical carcinoma and identified as a Δ^5-androstenetriol-3(β),16,17. The triacetate (m.p. 189.5–191°, $[\alpha]_D = -102°$), a diacetate (m.p. 183–187°), and the 3-monoacetate (m.p. 243–245°) of this compound as well as the dihydro derivative (androstanetriol-3(β),16,17, m.p. 256–260°) and its triacetate (m.p. 175.5–176.5°, $[\alpha]_D = -44°$) have been prepared.

The structural assignment is based chiefly on the conversions of the saturated triol $C_{19}H_{32}O_3$ into 3-ketoetioallobilianic acid and of the monoacetate of the isolated compound into β-3-hydroxy-Δ^5-etiobilienic acid.

The preparation of 3-ketoetioallobilianic acid from isoandrosterone via the 16-benzylidene derivative is described.

The question of the precursor of androstenetriol $in\ vivo$ is briefly discussed.

The author wishes to express his indebtedness to Dr. Erwin Schwenk of the Schering Corporation, Bloomfield, New Jersey, who supplied the dehydroisoandrosterone used in the preparation of 3-ketoetioallobilianic and β-3-hydroxy-Δ^5-etiobilienic acids, to Dr. Frieda B. Hirschmann for a sample of 3-ketoallocholanic acid, to Mrs. Dorothy Leekley and to Ensign Dorothy B. Hood for the colorimetric determinations, and to Mr. William Saschek, Department of Biochemistry, College of Physicians and Surgeons, New York, for the microanalyses reported in this paper.

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THE ISOLATION OF MONONUCLEOTIDES AFTER HYDROLYSIS OF RIBONUCLEIC ACID BY CRYSTALLINE RIBONUCLEASE*

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Since the discovery by Jones (1) of a thermostable enzyme in the pancreas, capable of hydrolyzing ribonucleic acid without the release of either phosphoric acid or purine bases, the evidence as to the nature of its action has been extremely conflicting. Jones and Perkins isolated four mononucleotides from enzyme-treated nucleic acid and concluded that the action of the enzyme consisted in breaking nucleotide linkages only (2). Levene, however, was not successful in his attempts to repeat the experiments of Jones ((3) p. 312) and in a paper with Schmidt (4) reached the conclusion that "The function of the enzyme is that of a depolymerizing agent, limited to the dissociation of the tetranucleotides of high molecular weight into those of lower molecular weight."

A thermostable enzyme with properties identical with those of the impure extracts mentioned above was isolated and crystallized by Kunitz and provisionally named ribonuclease (5). Its action consisted in the liberation of free acid groups without the formation of free phosphoric acid, and in the formation of split-products not precipitable by glacial acetic acid and readily diffusible through collodion or cellophane membranes. Allen and Eiler confirmed the crystallization of the enzyme and showed that the increase in free acid groups after enzymic action approached 1 equivalent for each mole of ribonucleic acid used, assuming a value of 1286 for the molecular weight of ribonucleic acid (6). Subsequently Bolomey and Allen showed that the hydrolytic action of a non-specific phosphatase was 50 to 150 per cent greater on ribonucleic acid that had been treated previously with ribonuclease in comparison with the untreated acid (7).

In the present paper we report the isolation and identification of four nucleotides from ribonucleic acid treated with crystalline ribonuclease, thus confirming the original finding of Jones. Control experiments on the fractionation of ribonucleic acid by similar procedures in the absence of enzyme treatment gave amorphous products with the general properties of the original nucleic acid.

As the term nuclease was originally suggested as generic for all enzymes which act on nucleic acid and their various split-products and nucleinase for those which disrupt polynucleotides to mononucleotides (8), it appears that the latter with the prefix ribo- to denote the type of nucleic acid is a more appropriate name for the crystalline enzyme. It is proposed, therefore, that the term ribonucleinase be adopted for this enzyme rather than ribonuclease as provisionally suggested by Kunitz.

<div align="center">EXPERIMENTAL</div>

Enzyme Preparations—In most of the experiments ribonucleic acid was hydrolyzed with either of two highly purified ribonucleinase preparations kindly provided by Dr. M. Kunitz.[1] One sample had been recrystallized five times from ammonium sulfate solution; the other four times from ammonium sulfate and three times from alcohol. Although all the nucleotides with the exception of adenylic acid were isolated after hydrolysis with these preparations, they were not obtained in a single experiment. In that in which all the nucleotides were isolated, as described in detail below, a sample of ribonucleinase prepared in this laboratory by the method of Kunitz (5) was used. It had been purified by three recrystallizations from ammonium sulfate solution and by one from alcohol.

Hydrolysis of Yeast Nucleic Acid—The method found most advantageous for the hydrolysis was the treatment of the ammonium salt of the ribonucleic acid at a neutral or slightly acid pH with ribonucleinase. In the experiment to be described the solution of nucleic acid was divided into two portions, one of which was treated with ribonucleinase and worked up for the fractionation of the products of hydrolysis. The other was worked up by the same procedure to determine whether similar products could be obtained from the neutral solution without the use of ribonucleinase.

100 gm. of commercial yeast nucleic acid (Eastman Kodak Company) were suspended in 400 ml. of distilled water, and concentrated ammonium hydroxide was added dropwise with mechanical stirring until the nucleic acid was completely dissolved. The pH of the solution was 6.3. The volume was adjusted to 500 ml. and the solution was divided into two equal portions. 50 mg. of crystalline ribonucleinase were added to one portion and both solutions were allowed to stand at room temperature for 18 hours. The pH of the enzyme-treated solution decreased to 5.5, while that of the control remained at 6.3. Both solutions were made neutral to litmus with ammonium hydroxide and were treated by Levene's method (9) for the fractionation of the nucleotides obtained by alkaline hydrolysis into guanylic and adenylic acid fractions, respectively.

[1] We should like to express our thanks to Dr. Kunitz for a generous supply of these products.

Identification of Nucleotides from Enzyme-Treated Ribonucleic Acid

Guanylic Acid Fraction—The guanylic acid fraction, consisting of the ammonium salts insoluble in 50 per cent alcohol, was reprecipitated from 50 per cent alcohol and converted to the corresponding brucine salts by Levene's method (9). After one recrystallization from 35 per cent alcohol, 2 gm. of a product which sintered at 185–195° and decomposed with evolution of gas at 218° were obtained. For further purification the brucine salt was converted to the crystalline tertiary sodium salt. 1.7 gm. of brucine salt were suspended in 10 ml. of 2 N sodium hydroxide, and the liberated brucine was extracted with chloroform. The clear aqueous solution was treated immediately with 40 ml. of 95 per cent alcohol, as given by Steudel and Peiser (10) for the preparation of trisodium guanylate. A yellow oil separated, which crystallized after it had been seeded with crystalline sodium guanylate and allowed to stand in the refrigerator overnight. This product was further purified by precipitation as the disodium salt from hot 20 per cent sodium acetate solution. It was then crystallized as before, as the trisodium salt by the addition of alcohol to the disodium salt dissolved in 2 N sodium hydroxide. The yield of twice crystallized sample was 0.15 gm. In another experiment in which 100 gm. of ribonucleic acid were hydrolyzed and the precipitation from 20 per cent sodium acetate solution was omitted, 3.8 gm. of a sample of trisodium guanylate, crystallized three times, were obtained. The specific rotation of the dried product (110° to constant weight over phosphorus pentoxide *in vacuo*) in 5 per cent sodium hydroxide was $[\alpha]_D^{21} = -56.1°$ (*c*, 0.8 per cent). The specific rotation of crystalline trisodium guanylate prepared by alkaline hydrolysis of yeast nucleic acid and determined under the same conditions was $[\alpha]_D^{23} = -57.6°$.

Preparation of Pure Dibrucine Guanylate—0.11 gm. of the sodium guanylate described above obtained by enzyme treatment was converted directly to the brucine salt by adding 1 ml. of 1 N acetic acid and 0.18 gm. of brucine dissolved in alcohol. When the hot solution had cooled, the crystalline dibrucine salt separated. After two recrystallizations from 35 per cent alcohol the yield of air-dried product was 0.1 gm. This product, like pure dibrucine guanylate, when placed in the melting point bath at 200° sintered at 210° and decomposed with evolution of gas at 224°. A mixed melting point determination with pure dibrucine guanylate showed exactly the same behavior. Analysis of the air-dried compound for nitrogen by the micro-Dumas method gave 9.97 and 9.95 per cent as compared to the theoretical of 9.85 per cent nitrogen for $C_{10}H_{14}O_8N_5P(C_{23}H_{26}N_2O_4)_2 \cdot 7H_2O$.

Fractionation of Adenylic Acid Fraction. Uridylic Acid—The ammonium salts soluble in 50 per cent alcohol were converted to brucine salts by the

usual procedures. These were recrystallized nine times from 35 per cent alcohol as employed for the purification of dibrucine uridylate. 2.7 gm. of material which sintered at 171–178° and decomposed with frothing at 212–217° were obtained from 50 gm. of yeast nucleic acid. Analysis of the air-dried compound by the micro-Dumas method gave 6.63, 6.87, and 6.51 per cent nitrogen as compared to the theoretical of 6.79 per cent for $C_9H_{13}O_9N_2P(C_{23}H_{26}O_4N_2)_2 \cdot 7H_2O$. The specific rotation of the air-dried compound in dry pyridine was $[\alpha]_D^{23} = -54.4°$ (c, 1.16 per cent). The value for dibrucine uridylate under the same conditions is $-55.9°$ (11). Pure dibrucine uridylate was obtained after enzymic hydrolysis in several other experiments; in one in which the yield was determined, 4 gm. of a sample crystallized nine times were isolated from 100 gm. of ribonucleic acid.

Further identification of uridylic acid was obtained by converting the dibrucine salt to the crystalline diammonium salt by the method of Levene (12). 0.83 gm. of thrice crystallized diammonium uridylate was recovered from 5.5 gm. of brucine salt. When placed in the melting point bath at 165°, it shrank at 170–175° and decomposed with evolution of gas at 183°. A mixed melting point determination with a sample of diammonium salt prepared by alkaline hydrolysis showed the same behavior. The specific rotation of the air-dried compound in water was $[\alpha]_D^{24} = +20.9°$ (c, 2 per cent) as compared to the value of $[\alpha]_D = +21°$ found by Levene (12).

Isolation and Identification of Cytidylic Acid—The mother liquors obtained from the first three crystallizations of the adenylic acid fraction described above were combined and concentrated to a small volume *in vacuo* at 40°. The brucine salts which separated (45 gm.) were recrystallized three times from 35 per cent alcohol, and the resulting mother liquors were again concentrated to a small volume *in vacuo* at 40°, giving another fraction of brucine salts (37 gm.). The latter brucine salts were converted to the corresponding free acids by way of the ammonium and lead salts, and the solution of the free acids was concentrated to a syrup in a vacuum desiccator over sulfuric acid. Attempts to cause crystallization by dissolving the syrup in small volumes of water and taking to dryness in a desiccator were finally successful, and a crystalline product was obtained which could be recrystallized by Levene's method for cytidylic acid (13). After five crystallizations 0.4 gm. was obtained. When placed in the bath at 218°, this product decomposed sharply at 230° (corrected) with evolution of gas, and the decomposition point was not lowered when mixed with known cytidylic acid. The air-dried compound was free from water of hydration and in two analyses gave 12.56 and 12.97 per cent nitrogen (micro-Dumas) as compared to the theoretical of 13.00 per cent for $C_9H_{14}O_8N_3P$.

The most insoluble brucine salts obtained from the first three mother

liquors as described above and the brucine salts from the mother liquors of the four to nine crystallizations of the brucine uridylate fraction were converted to the corresponding free acids. From a total of 35 gm. of brucine salt obtained in several different experiments, 2.8 gm. of twice crystallized cytidylic acid were isolated.[2]

Isolation and Identification of Adenylic Acid—The mother liquors from the first three crystallizations of the cytidylic acid mentioned above obtained from the most soluble brucine salts were combined and concentrated to a small volume *in vacuo* at 40°. The solid which separated on the addition of alcohol was redissolved in water and reprecipitated four times with alcohol. It was then extracted three times at 0° with dry pyridine. The residue was dissolved in a small volume of warm water by the addition of ammonium hydroxide to a neutral reaction, and the solution was made acid to Congo red with hydrochloric acid and allowed to cool slowly. 210 mg. of crystals separated with the characteristic appearance of adenylic acid prepared by alkaline hydrolysis. After two more crystallizations as described above and after one from hot water, 100 mg. of a product which decomposed at 192° were obtained. A mixed melting point with known adenylic acid gave the same value. The specific rotation of the air-dried compound in water was $[\alpha]_D^{24} = -38°$ (*c*, 1 per cent). The rotation of adenylic acid prepared by alkaline hydrolysis and determined under the same conditions was $[\alpha]_D^{24} = -39°$. The sample obtained after enzymic hydrolysis contained 17.1 per cent nitrogen as compared to the theoretical of 19.23 per cent for $C_{10}H_{14}O_7N_5P \cdot H_2O$. As adenylic acid sometimes contains cytidylic acid as an impurity (14), the sample was recrystallized under the conditions used to purify cytidylic acid, and the mother liquors were again worked up for adenylic acid. 25 mg. of long needles having a decomposition point of 196° (corrected) were obtained. This product contained 18.08 per cent nitrogen. While the analysis was somewhat low for pure adenylic acid, the sample agreed in decomposition point, optical activity, and crystalline form with yeast adenylic acid prepared by alkaline hydrolysis. Because of the small amount of sample remaining at this point, further purification was not attempted.

In the preparation of adenylic acid after alkaline hydrolysis, the free acid is usually obtained from the mother liquors of the first three crystallizations of the brucine salts of the adenylic acid fraction. Several attempts in other experiments to work up similar fractions for adenylic acid after enzymic hydrolysis always led to the isolation of cytidylic acid. Adenylic acid was finally obtained in the preparation described with great difficulty and in extremely small yield by the procedure mentioned above.

[2] These fractions were worked up by John G. Pierce in connection with certain experiments in which a source of cytidylic acid was required.

Attempted Fractionation of Control

The control, consisting of 50 gm. of the ammonium salt of ribonucleic acid, was treated with alcohol as carried out for the fractionation of hydrolyzed nucleic acid into guanylic acid and adenylic acid fractions respectively. The part which separated from 50 per cent alcohol weighed 26 gm. The ammonium salt soluble in 50 per cent alcohol (24 gm.) was converted to the free acids by preparing the lead salts and treating the latter with hydrogen sulfide. On neutralization of the aqueous solution with brucine, a brown gummy material separated. Repeated attempts to crystallize this gum from 35 per cent alcohol failed. Although no quantitative experiments were carried out, the general behavior of this material was comparable to that of the brucine salt obtained when ribonucleic acid was neutralized directly with brucine. The yield of the amorphous brucine salt dried in a vacuum desiccator over sulfuric acid was 25 gm.

DISCUSSION

The isolation of mononucleotides after hydrolysis of ribonucleic acid by the crystalline ribonuclease of Kunitz or as suggested above by ribonucleinase establishes the mode of action of this enzyme and is in agreement with the earlier results of Jones and Perkins with less pure enzyme preparations. The conflicting findings of Schmidt and Levene are based on the observations that the products of digestion were not dialyzable through cellophane, as was the mixture of mononucleotides obtained by alkaline hydrolysis, and that no appreciable change was found in depression of the freezing point of the digested nucleic acid. It should be pointed out, however, that the dialysis after alkaline hydrolysis was carried out in the absence of the enzyme preparation which probably contained appreciable amounts of inert protein, and that the conditions for the two experiments were therefore not comparable. Furthermore Kunitz (5) has demonstrated that about 50 per cent of the phosphorus present after treatment of ribonucleic acid with crystalline ribonucleinase dialyzes through cellophane. It seems established, therefore, in agreement with the isolation of the mononucleotides that at least a portion of the original nucleic acid is diffusible after enzyme action. Isolation procedures such as are available for the preparation of the nucleotides obviously cannot provide a quantitative answer to the question as to the amounts of mononucleotides produced. The experiments of Kunitz and those of Allen and Eiler, however, give some measure of this. In quantitative experiments, Kunitz found that a maximum of about 40 per cent of the original ribonucleic acid was no longer precipitable by uranium acetate after treatment with ribonucleinase. As mononucleotides were not precipitated under the conditions used (15), this gives a maximum of 40 per cent for the amount of mononucleotides which

could have been produced during the hydrolysis. Allen and Eiler showed that the increase in free acid groups after enzymic action approached 1 equivalent for each mole of ribonucleic acid used, assuming a molecular weight of 1286. If the original ribonucleic acid is assumed to be an asymmetric polynucleotide, as indicated by several lines of evidence (16, 5, 17), and the end-products of ribonucleinase hydrolysis are mononucleotides, it is possible to calculate the percentage of mononucleotides formed to account for the increase in acidity. Such calculations give a minimum of 25 per cent for a highly asymmetric polynucleotide molecule with values increasing, as the molecular weight is decreased, to 33 per cent for a single tetranucleotide of the structure postulated by Levene. As other products intermediate in size and relative acidity between the original nucleic acid and mononucleotides are also probably formed, the percentage of the latter present after hydrolysis would probably be even smaller than the lowest value. The above calculations show a surprising agreement between the results of Kunitz and those of Allen and Eiler on the assumption that the end-products of ribonucleinase action are mononucleotides and are in accord with the relatively small yields isolated in the present experiments. The presence of mononucleotides in the digestion mixture also accounts for the greater hydrolytic action of phosphatase on ribonucleinase-treated nucleic acid as found by Bolomey and Allen.

SUMMARY

The isolation and identification of four mononucleotides, guanylic acid, uridylic acid, cytidylic acid, and adenylic acid, after hydrolysis of yeast nucleic acid by the crystalline ribonuclease of Kunitz are reported. That the mononucleotides were not formed during the fractionation procedures was shown by the failure to obtain mononucleotides in control experiments in which nucleic acid, in the absence of enzyme, was fractionated under the same conditions.

In conformity with the nomenclature of Levene and Medigreceanu for enzymes which hydrolyze nucleic acid to mononucleotides, it is suggested that the name ribonuclease, which was provisionally given to the enzyme before the nature of the split-products had been determined, be changed to "ribonucleinase."

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A NEW METHOD FOR THE ISOLATION OF CRYSTALLINE ADENINE NUCLEOTIDES*

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Interest in the chemistry of the nucleic acids, their decomposition products and related compounds, has been stimulated by several important findings during the past decade: (*a*) the occurrence of desoxyribonucleic acid (thymus nucleic acid) in the nuclei of *yeast* cells (1); (*b*) the occurrence of ribonucleic acids (yeast nucleic acid) in the *cytoplasm* of rye embryos (2); (*c*) the serological activity of the nucleic acids and their decomposition products (3, 4); (*d*) the function of certain mono- and dinucleotides as coenzymes in reactions in intermediary metabolism too numerous to mention; (*e*) the effect of certain adenine compounds, in combination with other factors, in reproducing on yeast cells the proliferation-producing effect of products derived from damaged cells (5).

Among the simple purine nucleotides, the adenine compounds appear to be of prime physiological importance. Adenine nucleotide (9-adenine-*d*-furanoriboside-3-phosphoric acid) was first prepared in crystalline form in 1919 by Jones and Kennedy (6). Muscle adenine nucleotide or adenylic acid (9-adeninefuranoriboside-5-phosphoric acid) was first isolated in 1927 by Embden and Zimmermann (7). Both of these compounds have since been prepared by other investigators but they have not been readily available in pure crystalline form, even for experimental work, because of the tremendous cost and low yields which were incident to the non-specificity of the reactions employed in their isolation.

In this paper there is described a new reaction which has been employed as the key in simple procedures for the isolation of both of these adenine nucleotides in pure crystalline form and in relatively good yield.

EXPERIMENTAL

New Reaction

When, to a dilute solution of adenine nucleotide, there was added singly either a solution containing picrate ions or a solution containing aluminum ions, no precipitate was formed. When both of these reagents were added simultaneously in suitable amounts to such a solution, there appeared a

highly insoluble, light, semicrystalline, bright yellow precipitate, not unlike adenine picrate in macroscopic appearance. The "yellow precipitate" was formed in good yield in acid solutions when the pH of the solution was 2.4 or greater, either in the presence or absence of such ions as sodium, potassium, chloride, sulfate, acetate, etc., although these ions, when present, entered into the complex precipitate formed. The precipitate appeared to consist essentially of an aluminum picrate complex of adenine nucleotide.

The decomposition of the yellow precipitate was accomplished most conveniently by its solution in morpholine, followed by the precipitation of the aluminum salt of adenine nucleotide upon the addition of acetone.

Isolation of Adenine Nucleotide from Yeast Nucleic Acid

The separation of adenine nucleotide from the pyrimidine nucleotides : · a suitable hydrolysate of yeast nucleic acid presented no problem, since the latter formed neither insoluble aluminum salts nor insoluble aluminum picrate complexes. Because of the relative insolubility of the simple aluminum salt of guanine nucleotide, however, it was found convenient to remove the guanine nucleotide prior to the precipitation of the adenine nucleotide as the yellow compound.

Hydrolysis of Yeast Nucleic Acid—20 gm. of commercial yeast nucleic acid[1] were suspended in 500 ml. of water. Solid pellets of potassium hydroxide were added until the nucleic acid dissolved and the reaction was neutral to litmus. Enough more potassium hydroxide was added to make the solution approximately 0.3 N with respect to KOH[2] and the solution was allowed to stand for 24 hours at room temperature.

Glacial acetic acid was then added until the solution was neutral toward litmus, and the volume of the solution was noted.

Precipitation of Guanine Nucleotide—The potassium acetate salt of guanine nucleotide was precipitated by the addition of an equal volume of 95 per cent ethanol.[3] The precipitate was allowed to stand for several hours before it was collected by centrifugation. The precipitate was either discarded or used for the preparation of guanine nucleotide according to the method of Buell and Perkins (10).

Precipitation of Aluminum Picrate Salt of Adenine Nucleotide—Alcohol was removed from the filtrate obtained after separation of the guanine nucleotide fraction by concentration of the solution to a volume of about

[1] This preparation was kindly furnished by the Smith, Kline and French Laboratories, Philadelphia.

[2] This method of hydrolysis is an adaptation of the method of Steudel and Peiser (8).

[3] The separation of guanine nucleotide as the potassium acetate salt was first described by Jones and Perkins (9).

finally as the lead salt from which the free acid was converted easily into crystalline form.

Freezing the Muscle—25 pounds of carbon dioxide ice were sawed into small pieces which were added to 6 liters of acetone. To this freezing mixture there were added 11 pounds of muscle tissue prepared from four beef hearts, as follows: As soon as possible after each animal was killed the heart was cut out, and rapidly freed from adherent blood vessels, connective tissue, gross fatty tissue, etc., and the muscle was cut into narrow strips. These strips were then frozen rapidly and brought to the laboratory, where they were allowed to stand at room temperature until they were just soft enough to pass through an electric meat grinder.

Extraction and Deproteinization—To the cold, finely ground muscle there were added without delay 11 liters of water. The brei was stirred rapidly while live steam was passed through it for about 10 minutes until the boiling temperature was reached. After it had boiled vigorously for 1 minute, the mixture was allowed to cool spontaneously to 75° and was then treated with 180 gm. of picric acid (10 per cent moisture), stirred well, and allowed to stand for several hours with occasional stirring before it was filtered onto large fluted papers.

Precipitation As Mercury Salt—The filtrate, which was sparklingly clear, was treated with 240 gm. of mercuric acetate. After several hours the precipitated mercury salts were collected and washed once with generous quantities of warm water. They were then suspended in 2 liters of hot water, and were treated with hydrogen sulfide for 2 hours while the reaction mixture was kept warm and was shaken frequently. The mercuric sulfide was finally collected on a Buchner funnel, where it was washed thoroughly with warm water, the washings being added to the filtrate.

Precipitation As Aluminum Picrate Complex—To the aerated solution there were added 1500 ml. of aluminum picrate reagent. After several hours the yellow precipitate was collected at the centrifuge, washed, and decomposed with morpholine and acetone. The dry aluminum salt was put into solution in 2 liters of hot water, in which it was readily soluble without the addition of alkali. Aluminum hydroxide was precipitated by the careful addition of acetic acid to pH 5.

Final Collection and Purification of Product—The adenylic acid was collected as the lead salt, which was decomposed in the usual manner. On evaporation of the filtrate at 40° under reduced pressure, adenylic acid crystallized in the distilling flask, but, since the crystals had a yellow tinge which was not removed readily upon recrystallization, they were redissolved by warming them in the mother liquor and acetone was added as long as a precipitate formed. The precipitate was washed with acetone, dried in air, and dissolved in water, and the adenylic acid was precipitated once

more as the lead salt, which was washed and decomposed in the usual way. Care was taken to keep the volume of the solution small, since it was evaporated first at room temperature in a vacuum desiccator over sulfuric acid and finally in the ice box. After one recrystallization from hot water, 800 mg. of beautiful snow-white crystals were obtained which were microscopically indistinguishable from adenine nucleotide prepared from yeast nucleic acid.

The following results were obtained by microanalysis and compared with the theoretical, based on the empirical formula $C_{10}H_{14}N_5PO_7 \cdot H_2O$.

Calculated.　C 32.68, H 4.37, N 19.13, P 8.47, H_2O 4.91
Found.　　　"　31.96, "　4.53, "　19.14, " 7.99, "　4.79

DISCUSSION

Heretofore there has been available no simple characteristic reaction by means of which the adenine nucleotides might be separated easily from other compounds with which they occur in nature. For example, such devious means as nine recrystallizations of the brucine salts of the nucleotides derived from yeast nucleic acid have been used to effect the separation (11).

The new reaction described is not entirely specific for adenine mononucleotides. It has been used for the preparation of the polynucleotide known as yeast nucleic acid in sufficient purity to result in an occasional crystalline specimen. Under the microscope these crystals of yeast nucleic acid appear as long, tapering needles closely resembling adenine nucleotide in form. Theoretically, the new reaction should prove useful in the isolation of those dinucleotides in the structure of which adenine nucleotide is a component part, such as the adenine-pyridine dinucleotides and the adenine-alloxazine dinucleotides.

No claim is made for the absolute specificity of the new reaction, even as a group reaction, for the adenine mono- and polynucleotides. Obviously, any compound, the simple aluminum salt of which is relatively insoluble in acid, will appear in the "yellow precipitate" unless it is first removed from solution. Doubtless there are many compounds which will give the reaction; it is, nevertheless, sufficiently specific to serve as the key reaction in the isolation of the adenine nucleotides from their natural sources.

SUMMARY

1. A new reaction has been described which is characteristic of the adenine mononucleotides and also of yeast nucleic acid.

2. A method in which this reaction is utilized has been described for the isolation of crystalline adenine nucleotide from yeast nucleic acid. The

method is relatively simple, inexpensive, and rapid, and the yields obtained are gratifying.

3. A method in which this same reaction is likewise utilized has been described for the isolation of crystalline adenylic acid from beef heart. Essentially the same procedure has been used for the preparation of the compound from other tissues such as blood.

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N¹-METHYLNICOTINAMIDE, A METABOLITE OF NICOTINIC ACID IN THE URINE

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With Spectroscopic Data by D. G. Sharp

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Najjar and coworkers (1) have described a fluorescent material, designated F_2 by them, which appears in the urine of humans, in small amounts normally, and in larger amounts after the ingestion of nicotinic acid or nicotinamide.

In a preliminary note (2) data were presented showing the biological and chemical similarity between F_2 and N¹-methylnicotinamide. The latter substance was studied in detail in 1936, as a model for the newly discovered pyridine nucleotide coenzymes, by Warburg (3) and Karrer (4) and their collaborators. Data presented in this communication demonstrate the chemical identity of these two substances.

EXPERIMENTAL

Isolation of F_2 from Urine—Urines were collected from two normal individuals who had ingested 3 gm. of nicotinamide over a period of 3 days. 8 liters of urine, acidified with acetic acid and cleared with charcoal, were evaporated to 400 ml. on the water bath in a current of air. The mushy residue was extracted with 1 liter of 95 per cent ethanol (redistilled to remove fluorescent materials). The alcoholic extract was evaporated and the residue reextracted with 80 per cent ethanol. The residue after evaporation of this extract was taken up in 2 liters of water, adjusted to pH 4 with acetic acid and sodium acetate. The solution was passed slowly (1 drop per second) through two columns, 42 cm. long and 2.5 cm. in diameter, containing 100 gm. of 60 to 80 mesh Decalso,[1] previously washed with acetic acid, KCl, and water. Tests indicated that only 30 per cent of the F_2 originally present in the urine was adsorbed on the permutit. Therefore, after the elution with 275 ml. of 25 per cent KCl solution and washing with water the urine extract was passed through the columns twice with elution and washing after each passage. A total of 650 mg. of the 900 mg. of F_2

[1] A zeolite obtained from The Permutit Company, 330 West 42nd Street, New York.

estimated to be present in the original urine was found to be in the above three KCl eluates by direct determination.

The combined eluates, about 700 ml., were evaporated in an air current on the steam bath almost to dryness, and the KCl residue was extracted with 90 per cent alcohol, reevaporated, and the procedure repeated several times in diminishing volumes of 95 per cent alcohol until the final extract in 20 ml. of 95 per cent ethanol was virtually free of KCl. To this extract a calculated excess of picric acid in a saturated alcoholic solution was added, and the solution was warmed to 60°, slowly cooled, and finally chilled in a salt-ice mixture. The cold crystalline mass was rapidly filtered off with suction and washed with cold 95 per cent alcohol and cold ether until free of picric acid. The material was recrystallized twice from 95 per cent alcohol. The melting point of the hexagonal prismatic crystals was 189.5° (uncorrected).

The picrate was decomposed by suspending the crystals in 4 ml. of water containing a calculated slight excess of HCl and the mixture was repeatedly shaken with washed dry ether, until no more color was taken up. The aqueous acid solution was evaporated to dryness from a test-tube at room temperature with a current of nitrogen. The residue was dissolved in the minimal volume of boiling absolute ethanol and the crop of crystals obtained on slow cooling, finally in ice.

The yield was 120 mg. of the product out of the initial content of 900 mg. in the urine as calculated from the fluorescence analyses for F_2. This yield may be conceivably increased by improvements in the technique, particularly by employing NaCl instead of KCl for elution, since the sparingly soluble K picrate is difficult to separate from the picrate of the base.

The synthetic N¹-methylnicotinamide was prepared[2] from nicotinamide by heating under a reflux for 1 hour at 42° with a 50 per cent excess of methyl iodide in methyl alcohol. The excess methyl iodide and the alcohol were evaporated. The yellow crystalline mass was recrystallized twice from hot methyl alcohol. The iodide was changed into the chloride by shaking an aqueous solution with freshly prepared AgCl as recommended by Karrer et al. (4). The resulting N¹-methylnicotinamide chloride obtained by evaporation in vacuo was crystallized from methanol. The melting point as given by Karrer is "about" 240°. Our synthetic product melted at 237–238° (uncorrected); the product from the urine melted at 233–234°, and the mixed melting point was at 230–232°. The melting point in each case was rather poorly defined.

A comparison of the composition and properties of the N¹-methylnicotinamide chloride and of the chloride of the urinary product is summarized in Table I.

The nitrogen was determined by steam distillation with strong NaOH into

boric acid and titration. Previous experience with compounds of this type showed that under these conditions the alkali hydrolyzes the —$CONH_2$ group to NH_3 and splits the $CH_3N^=$ group from the ring as CH_3NH_2, the yield of nitrogen being about 97 to 98 per cent of the theoretical.

The chloride was determined by titration with $AgNO_3$ and with dichlorofluorescein as indicator (5). The nicotinic acid content was determined by the method for trigonelline developed in these laboratories (6), which includes hydrolysis with 1.5 N HCl followed by hydrolysis with 6 N KOH in the presence of urea.

The measurement of fluorescence of the alkaline butanol extract was carried out by the procedure described below.

The absorption spectra as shown in Fig. 1 (determined by D. G. Sharp) were obtained with the aid of a Hilger quartz spectrophotometer, model

TABLE I

Comparison of Composition and Properties of Synthetic N^1-Methylnicotinamide Chloride and of Urinary F_2 Chloride

Product	N	Cl	Nicotinic acid	Fluorescence	Absorption maximum	M.p. of picrate
	per cent	*per cent*	*per cent*	*units[*] per γ*	*λ.*	*°C.*
Synthetic.....	15.83	20.45	70.5	3.04	2645	189.5
Urinary.......	15.68	20.60	71.1	3.02	2645	189.5
Theory.......	16.23	20.60	71.3			189.5 (mixed m.p.)

[*] Fluorescence units represent galvanometer divisions per microgram of substance, measured under the conditions described in the text.

E-369. For comparison, curves for the two closely related derivatives of nicotinic acid, trigonelline and nicotinamide, were included. Whereas the curves of N^1-methylnicotinamide chloride and of the chloride of F_2 show very close coincidence, those for trigonelline and for nicotinamide show significant divergence.

From the data presented in Table I and Fig. 1 and from our previous observations (2) it is apparent that the substance F_2 is identical with N^1-methylnicotinamide.[3]

[3] Both Karrer *et al.* (4) and Warburg and Christian (3) designated the compounds as *Nicotinsäureamid jodmethylat, Nicotinsäureamid chlormethylat, Iodmethylat des Nicotinsäureamids*, etc. It is proposed to designate the ion [structure] as N^1-
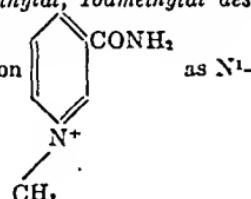
Since the work detailed above was largely controlled and facilitated by the fluorometric determination introduced by Najjar and collaborators (1), it is deemed desirable to outline briefly the simple, rapid, and convenient modification of the method employed by the present authors.

The adsorption at pH 4 on permutit columns was carried out much in the manner recently described for thiamine by Mason and Williams (7). The elution was performed with 25 per cent KCl solution at room temperature. The extraction from the KCl into the organic solvent is critical and must be carefully standardized. It was found that the distribution coef-
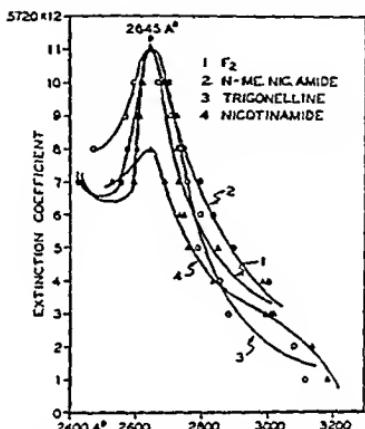
FIG. 1. Absorption curves of F_2 chloride isolated from urine (Curve 1), synthetic N¹-methylnicotinamide chloride (Curve 2), trigonelline chloride (Curve 3), nicotinamide (Curve 4), all in 0.175 M concentration in NaHCO₃-Na₂CO₃ buffer, pH 9.7; 1 cm. cell, Hilger quartz spectrophotometer, E-369. The figures on the ordinate multiplied by the factor 5720 give values of the molecular extinction coefficient, $\epsilon = \frac{1}{cl} \log_{10} \frac{I_0}{I}$.

ficient of F_2 between the aqueous and the butanol phases is greatly in favor of the water and follows a very steep curve. It was found convenient to select 5 ml. of 25 per cent KCl and 12 ml. of n-butanol as standard volume ratios. If other ratios are used, larger or smaller degrees of extraction will result. The extraction is conveniently carried out as follows:

5.0 ml. of the KCl eluate (or a smaller aliquot with the addition of 25

methylnicotinamide. Being a quaternary ammonium compound, it presumably exists in aqueous solution as a cation. Its full designation in terms of modern organic nomenclature would be N-methylpyridinium hydroxide-3-carboxylic acid amide (suggested by Professor L. A. Bigelow of Duke University). In crystalline form, it has been isolated as the iodide, chloride, and picrate. "Trigonellamide" is another possibility for an abbreviated name (suggested by Dr. H. P. Sarett).

per cent KCl solution to bring the volume to 5.0 ml.) are placed in a 125 ml., glass-stoppered, conical separatory funnel, followed by 12.0 ml. of n-butanol.   The mixture is briskly stirred with a current of air, and 1.0 ml. of 10 N NaOH is blown in rapidly with the aid of a rubber bulb attached to the pipette.   The stirring is continued for 1 to 1.5 minutes, the aqueous layer is drawn off, and the butanol is cleared by shaking with 2 gm. of powdered anhydrous Na_2SO_4.   The decanted clear solution is read after 20 minutes in a suitable instrument.   We found the Coleman electric fluorophotometer, model 12, equipped with Filters B-1 and PC-1 (for thiochrome determinations) entirely satisfactory.   The instrument is standardized by setting the galvanometer at 100 with a solution containing 0.3 γ of quinine sulfate per ml. in 0.1 N H_2SO_4.   The butanol extract prepared as described above from a solution containing 10 γ of N^1-methylnicotinamide, or 10 γ of urinary F_2, gives a scale reading of 30 divisions, or 3 divisions for 1 γ.   This value is subject to small changes with fluctuations in the instrument, and it is easily checked with a standard solution run with each set of determinations.   The reagent blank usually amounts to 10 divisions and should be ascertained.   The accuracy of the determination with 10 to 20 γ samples is within 5 per cent.   For smaller amounts the error may be greater, but 5 γ may be determined with an accuracy of 10 per cent.   It is worth noting that the Decalso columns may be used over repeatedly if washed with about 500 ml. of water after each elution with KCl and stored immersed in a cylinder of water.

Values obtained by all of the previously described methods for the determination of "trigonelline" in urine either by conversion to nicotinic acid (6, 8) or directly by condensation with an aromatic amine as in Kodicek and Wang's method (9, 10) must necessarily include the N^1-methylnicotinamide, in so far as varying degrees of acid or alkaline hydrolysis which converts this compound to trigonelline are employed in all of these methods.   The implications of this fact have been discussed in part by Sarett (10) in the recent description of his modification of the Kodicek and Wang method and will be discussed in greater detail in a forthcoming publication.   The data on hand indicate, indeed, that, in man and in the rat, doses of either nicotinic acid or of its amide result in the excretion of N^1-methylnicotinamide, as the chief end-product, rather than of trigonelline.

SUMMARY

A crystalline substance was isolated from human urine after dosage with nicotinamide.   From comparison with synthesized N^1-methylnicotinamide chloride in regard to content of nitrogen, chloride, and nicotinic acid, and in regard to the absorption spectra, fluorescence, and the melting point of the picrates the two substances were found to be identical.   This es-

tablishes the identity of the fluorescent substance, F$_2$, previously described
by Najjar, Holt, and their collaborators.

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THE METHYLATION OF NICOTINAMIDE BY RAT LIVER IN VITRO*

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During recent years several attempts made by workers in these laboratories to demonstrate the methylation of nicotinic acid or of its amide *in vitro* by tissues of animals which were known to excrete "trigonelline" after doses of these compounds were unsuccessful. This failure was ascribed largely to the technical difficulty of determining very small amounts of trigonelline in the presence of a relatively large excess of unused nicotinic acid, for the only method for the determination of trigonelline was based on its transformation into nicotinic acid by means of alkaline hydrolysis in the presence of urea (1) and the measurement of the increment in the total nicotinic acid present. The method of Kodicek and Wang (2) for the direct determination of "trigonelline" appeared inadequate and inaccurate in the preliminary form in which it was published. In recent months, Fox, Mc-Neil, and Field (3) and also Sarett (4) elaborated more workable modifications of this method. At about the same time, it evolved that the urinary fluorescent material, F_2, of Najjar and collaborators (5) is identical with N^1-methylnicotinamide (6, 7). Our studies (7) indicate, furthermore, that at least a large proportion of the metabolite appearing in the urine after the ingestion of nicotinic acid or of its amide which was hitherto designated as "trigonelline" consists in fact of the amide of trigonelline, or of N^1-methylnicotinamide. It was, therefore, decided to reinvestigate this problem by means of the newer methods for determining the methylated derivatives of nicotinic acid.

EXPERIMENTAL

Mature rats of both sexes, of the Vanderbilt strain, were killed by decapitation and exsanguinated. The livers were removed immediately, and slices, 0.2 to 0.5 mm. thick, were made by hand, washed in two changes of Ringer's solution, blotted, and 150 to 450 mg. weighed rapidly on a torsion

balance, and the slices placed in 50 ml. Erlenmeyer flasks with 4.0 ml. of Ringer-bicarbonate solution (as used by Krebs and Henseleit (8)) and substrate substances; the solution was equilibrated with a mixture of 95 per cent O_2 and 5 per cent CO_2; the flasks were stoppered and agitated in a Warburg bath at 37° for varying periods of time. In the anaerobic experiment, the gas space was equilibrated with a mixture of 95 per cent N_2 and 5 per cent CO_2. The controls and experimental combinations were run in duplicate or triplicate. Immediately upon removal from the bath, the supernatant fluids were transferred, with small washings of water, to 15 ml. graduated tubes to a volume of 4.5 to 5.0 ml. and 0.5 ml. of 20 per cent trichloroacetic acid was added. The whole was mixed, heated in a water bath at 75–80° for 30 minutes, cooled, and made up to 5.5 ml. with water. The precipitated proteins were removed by centrifugation, and the supernatant fluid was used for fluorescence or colorimetric analyses directly. Adsorption on permutit and elution for fluorescence analysis were omitted after it was ascertained that the tissue extracts, unlike urine, contained relatively small concentrations of interfering pigments. In each experiment the control value for tissue slices alone without substrate was determined, and the increment due to the substrates was obtained by subtracting the control value. Added N^1-methylnicotinamide and trigonelline were recovered quantitatively under the above conditions.

The fluorescence analysis for F_2 (N^1-methylnicotinamide) was carried out essentially by the modified (7) procedure of Najjar et al. (5) except that the transfer of F_2 from the aqueous to the butanol phase was further facilitated by dissolving 1.8 gm. of anhydrous Na_2SO_4 in the 5 ml. aliquots of the tissue extracts. This salting-out procedure was found to result in an increase of the transfer of F_2 to the butanol phase, so that the galvanometer reading obtained was 5 to 6 divisions per microgram of F_2 present in the aqueous phase, as compared with a reading of 3 divisions per microgram obtained in the transfer from 25 per cent KCl solution, as used in the case of eluates from urine. Unfortunately, concentrated Na_2SO_4 solutions cannot be successfully used at room temperatures below 25° without troublesome crystallization and resulting diminution of the transfer of F_2 to the butanol.

The procedure is briefly as follows: 5.00 ml. of the protein-free tissue extract are measured into 125 ml. conical separatory funnels, 1.8 gm. of anhydrous Na_2SO_4 are added and dissolved by agitation or with the aid of the pipette carrying the air current which is used for stirring as described below, and 12.0 ml. of n-butanol are added. While the two liquids are being stirred with a brisk air current passing through them, 1 ml. of 10 N NaOH is blown into the emulsion with the aid of a rubber bulb at the end of the pipette. The air current is continued for 1 to 1.5 minutes after the addition of the alkali and stopped. The aqueous phase is drained off after a brief period of separation; the butanol extract is shaken with about 2 gm.

of anhydrous Na_2SO_4 and decanted into the test-tubes or cuvettes and read at once in the fluorophotometer and again after standing 15 to 20 minutes in the dark.   In this study, we used the Coleman electric fluorophotometer, model 12, equipped with the two filters designed for the determination of thiochrome.

A summary of the data is presented in Table I.   These recorded observations demonstrate conclusively that rat liver slices methylate nicotinamide readily to N^1-methylnicotinamide, since the fluorescence method used does not include trigonelline and presumably is specific for N^1-methyl-

TABLE I

Synthesis of N^1-Methylnicotinamide by Rat Tissues in Vitro

Time of incubation, 3 to 4 hours.

Tissue	No. of experiments	Weight taken	Substrate	Range of increment*	Remarks
		mg.		$\gamma\ F_2\ per\ gm.\ tissue$	
Liver	20	120–450	Nicotinamide, 1.0 mg.	10–46 (25)	
"	5	200–400	Nicotinamide, 1.0 mg., + methionine, 2 mg.	23–45 (37)	25–50% increase over corresponding experiments with nicotinamide alone
"	5	200–400	" "	8–45 (24)	No increase over amide alone
"	6	200–350	Nicotinic acid, 1.0 mg.	0– 4	Increments too low to be significant
Kidney	2	220–240	Nicotinamide, 1.0 mg.	0– 3	
"	2	250–330	Nicotinic acid, 1.0 mg.	0	
Muscle, diaphragm	1	225	Nicotinamide, 1.0 mg.	0	

* The figures in parentheses are averages.

nicotinamide.   In several experiments parallel determinations were kindly made by Dr. Sarett on the liver extracts after incubation with nicotinamide by his modification of Kodicek and Wang's method (4) for total methylated nicotinic acid derivatives.   The values so obtained corresponded, within ±10 per cent, to those obtained by fluorescence analysis, indicating that no appreciable amounts of trigonelline were formed by liver slices from nicotinamide.   In experiments with liver slices from twenty-two rats not a single negative result was obtained.

The process appears to be strictly aerobic, for in the incubation of liver slices with nicotinamide in an atmosphere of nitrogen no trace of the methyl-

ated product could be found. That the intact cells are required was shown likewise by totally negative results when broken cells (liver brei) were employed in the presence of oxygen.

Kidney and diaphragm muscle slices produced with nicotinamide insignificant amounts, or none at all, of the methylated compound. The same was true of rat brain brei.

Nicotinic acid on incubation with liver or kidney slices did not yield significant amounts either of N^1-methylnicotinamide or of trigonelline and other methylated derivatives, as shown by Dr. Sarett's analyses in which his method (4) referred to above is employed.

Methionine when added to nicotinamide incubated with liver slices increased the methylation product by 25 to 50 per cent above that with nicotinamide alone in five out of ten experiments, whereas in five experiments this effect was completely absent. The same inconstancy of result was observed by Handler and Bernheim (9) in their study of creatine formation by liver slices in the presence of methionine.

Choline, when substituted for methionine, enhanced the methylation of nicotinamide in one experiment but failed to do so in another. It is planned to obtain more definite data on this aspect of the problem.

The time factor in the methylation of nicotinamide by liver slices *in vitro* at 37° is illustrated by the following values obtained with 370 to 380 mg. of liver incubated with 0.1 mg. of nicotinamide: 1 hour 3 γ, 2 hours 7.5 γ, 3 hours 12 γ, 4 hours 15 γ, per 1 gm. of liver. Methylation apparently proceeds at a fairly constant rate during the first 3 hours and slows up thereafter.

Attempts to study the effect of the concentration of the enzyme involved in this process by varying the weight of liver slices taken from 120 to 450 mg. gave somewhat erratic results which cannot be interpreted. Varying the concentration of the substrate (nicotinamide) in the presence of 200 mg. of liver slices gave the maximal effect with 0.25 mg. which was not increased by raising the amount of nicotinamide to 2.0 mg. in 4 ml. of Ringer's solution.

The stability of the methylation product in the system was indicated by the quantitative, 96 to 100 per cent, recovery of 100 γ of N^1-methylnicotinamide added to 200 to 400 mg. of liver slices in 4 ml. of Ringer's solution, agitated at 37° for 4 hours. Nor was trigonelline destroyed under these conditions. An illustrative experiment gave the following values.

	Galvanometer units	Increments
240 mg. liver control	22	
240 " " + 10 γ N^1-methylnicotinamide	75	53
Reagent blank	11	
10 γ N^1-methylnicotinamide	66	55

It has been repeatedly observed in the studies on the metabolism of nicotinic acid in rats[1] that individual rats vary widely in their daily excretion of methylated nicotinic acid compounds when maintained on ordinary nicotinic acid-free diets. Two such rats showing a very wide difference in excretion after a 3 mg. dose of nicotinamide were sacrificed and their livers tested with nicotinamide with and without added methionine. The results are shown in Table II. Apparently the rate of excretion of the methylated product parallels the rate of its formation in the liver.

DISCUSSION

Another methylation system has been demonstrated for rat liver *in vitro*: nicotinamide to N^1-methylnicotinamide, in addition to the established process of creatine formation (10). In the absence of this mechanism in rat kidney and muscle, in its aerobic character, and in the rôle played by methionine it resembles the creatine system, thus making it likely that the methylation process in the animal tissues follows a well defined pattern.

TABLE II

Excretion and Formation in Liver of Methylated Nicotinic Acid Compounds by Rats

Rat	F_2 in 24 hr. urine	F_2 produced per gm. liver	
		Liver + nicotinamide	Liver + nicotinamide + methionine
	γ	γ	γ
C	1030	32	40
D	355	17	23

It has been shown by Handler and Dann (11) that the feeding of large amounts of nicotinamide to rats is capable of inhibiting their growth by exhausting the supply of labile methyl groups, since the inhibition was overcome by methionine. It may now be presumed that the "trigonelline" determined in the urine of their rats was really N^1-methylnicotinamide.

The methylation of nicotinamide, but not of nicotinic acid, by liver slices raises the obvious question of the site of transformation *in vivo* of nicotinic acid into its amide prior to methylation. Recent observations in these laboratories[1] show that individual rats excrete almost identical amounts of the methylated amide in 24 hours after ingestion or parenteral injection of small amounts (3 mg.) of either nicotinic acid or nicotinamide. The problem as to whether the rat does or does not methylate nicotinic acid to trigonelline must also await solution in further experimentation. The data at hand indicate[1] that most, if not all, of the methylated products in the urine of rats on nicotinic acid-free diets are in the form of methylated

[1] Huff, J. W., and Perlzweig, W. A., unpublished data.

nicotinamide. After a large dose of nicotinic acid, however, there does appear to be, in certain cases, a considerable fraction of the total methylated product which is not the amide. This problem is being studied further.

SUMMARY

Rat liver slices when incubated with nicotinamide at 37° were shown to synthesize N^1-methylnicotinamide (F_2). This process is strictly aerobic, requires unbroken cells, and is usually, but not always, enhanced by the addition of methionine. Liver slices do not methylate nicotinic acid. Rat kidney and muscle do not exhibit, *in vitro*, the capacity to methylate nicotinamide. Suggestive evidence is presented to indicate that the individual variations in the rate of excretion of N^1-methylnicotinamide in the urine of rats are possibly related to the rate of methylation of nicotinamide in their livers.

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FERRITIN

VI. CONVERSION OF INORGANIC AND HEMOGLOBIN IRON INTO FERRITIN IRON IN THE ANIMAL BODY. STORAGE FUNCTION OF FERRITIN IRON AS SHOWN BY RADIOACTIVE AND MAGNETIC MEASUREMENTS*

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The properties of ferritin, an iron-protein compound found in the liver, spleen, and marrow of mammals, including man, have been reported in previous papers (1–3). Ferritin consists of a protein fraction, apoferritin, linked together with micelles of a special type of colloidal ferric hydroxide, the crystals containing as much as 23 per cent of iron. There are three possible states of ferric iron; namely, those with 1, 3, and 5 unpaired electrons in the outer electron shell. Magnetic measurements (3) have shown that iron in the micelles of ferritin is present in the rarely occurring state of 3 unpaired electrons per iron atom. This special property makes it possible to distinguish magnetically the iron of ferritin from any other form of ferric iron. The very high iron content of ferritin suggests that it functions as a storage compound. We have attempted to demonstrate this function by following the fate of iron labeled with the Fe^{59} radioactive[1] isotope injected intravenously as ferric ammonium citrate, as well as the fate of labeled iron contained as a constituent of the heme of the red blood cells. At the same time it was of interest to determine whether by injection of an excessive amount of iron containing 5 unpaired electrons (ferric ammonium citrate) the iron could be converted into a ferritin possessing higher magnetic susceptibility values per iron atom than that of regular ferritin.

Methods

The detailed procedures for the isolation of ferritin have been previously described (4), the tissue being ground with an equal volume of water, heated to 80°, filtered, and the filtrate treated with 35 gm. of ammonium sulfate per 100 ml. of filtrate. The brown precipitate obtained was dialyzed against distilled water, producing a brown solution which contained the

Following injection as ferric ammonium citrate or liberation from red cells which were destroyed by the action of acetylphenylhydrazine, it has been demonstrated that such iron is used for the construction of new ferric hydroxide micelles of ferritin.

In the first experiment (Table I) 82 per cent of the iron injected as ferric ammonium citrate was shown to be taken up by the liver. The ferritin and non-crystallizable fraction (F + NCF) which was isolated contained 75 per cent of the iron of this fraction in the form of radioactive iron, showing a conversion of the ferric iron of the injected ferric ammonium citrate into ferritin iron. Magnetic[2] susceptibility determinations showed that the

TABLE II

Ferritin Formation from Hemoglobin Iron of Red Blood Cells (Dog 42-816)

	Total Fe	Total activity	Specific activity	Distribution of labeled Fe; total circulating activity after transfusion taken as 100 per cent	Distribution of labeled Fe; activity lost to circulation = 100 per cent	Per cent Fe which is radioactive
	mg.	counts per min.	counts per min. per mg. Fe	per cent	per cent	
Injected tagged blood (110 ml.)	64.2*	6720*	105*	100		
Circulating blood after transfusion....	324	6720	20.7	100		19.8
Circulating blood before death........	127	1050	8.3	15.6		7.9
Liver (335 gm.).......	138	3100	22.5	46.1	55	21.4
F + NCF............	72.4	1925	26.5			25.3
Ferritin.........	12.4	332	26.8			25.5
Spleen (89 gm.)......	87.5	1150	13.2	17.1	20	12.5
F + NCF.............	26.1	328	12.6			12.0

* Estimated.

injected ferric iron containing 5 unpaired electrons had been converted into ferric hydroxide containing 3 unpaired electrons. Thus none of the in-

[2] Ferric ammonium citrate is an ionic compound containing 5 unpaired electrons per iron atom. When injected into the organism, the neutral reactions of the body fluids may bring about some hydrolysis of this compound with the resulting production of some ferric hydroxide which need not have the same magnetic susceptibility as the injected iron (3). The essential fact, however, is that the labeled iron of this F + NCF fraction shows precisely the same values for the magnetic susceptibility per iron atom as is found in all horse, human, and dog ferritins that have thus far been measured. The value for the magnetic susceptibility of ferritin iron is unique and readily distinguishes this iron from all other iron compounds normally occurring in the organism.

jected material in its original state could be detected in the isolated ferritin fraction by the magnetic method. The twice crystallized ferritin (F) of the liver had the same specific radioactivity as the F + NCF fraction, indicating the iron to be essentially the same in both fractions. The identity of the iron in the F + NCF and F fractions as determined by the radioactivity method corroborates previous findings of their identity as determined by the magnetic susceptibility method (3).

In the second experiment (Table II) red cells containing tagged iron as a constituent of their hemoglobin were injected and the resulting mixed cells of the circulation were destroyed with acetylphenylhydrazine. The dog was sacrificed after 6 days. During this time interval not only destruction of red cells but also undoubtedly some regeneration had occurred (10). The liver was found to have taken up 46 per cent of this labeled iron originally present in the circulation. 25 per cent of the total iron present in the F + NCF and in the twice crystallized ferritin fractions of the liver was present as labeled iron. The specific radioactivities of the liver and its ferritin fractions were somewhat higher than the circulating specific activity of the circulating red cells following transfusion of the tagged cells. The figures indicate that in this instance there was some preferential conversion of injected hemoglobin iron into ferritin iron owing possibly to a more rapid destruction of the injected erythrocytes. Of the labeled iron formerly in the circulation following transfusion, 17 per cent was found in the spleen. That the specific activity of the splenic iron is lower than that of the liver is an indication that a relatively large amount of nonradioactive iron was present in this organ of this normal dog prior to the transfusion and subsequent destruction of red cells.

The form in which iron is introduced appears to determine the distribution of this element in the viscera (11). In the first experiment the soluble ferric ammonium citrate was removed from the circulation almost wholly by the liver, only 0.35 per cent of the labeled iron having been taken up by the splenic tissue. In the second experiment, however, following breakdown of the red blood cells and their subsequent phagocytosis by the cells of the reticuloendothelial system there is an appreciable uptake of the iron by the spleen as well as the liver.

SUMMARY

Iron in the form of ferric ammonium citrate when administered by vein to the dog is readily converted into ferritin iron in the liver.

Iron derived from hemoglobin of the circulating red blood cells following the destruction of the cells by acetylphenylhydrazine is in part, at least, converted to ferritin iron in the liver and spleen.

The body is able to convert injected ferric iron of the form containing

5 unpaired electrons to ferric iron of the form containing 3 unpaired electrons, characteristic of ferritin.

It is concluded that ferritin iron acts in the capacity of storage iron in the animal body.

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THE RÔLE OF CARBOXYL-LABELED ACETIC, PROPIONIC, AND BUTYRIC ACIDS IN LIVER GLYCOGEN FORMATION

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From experiments previously reported from this laboratory, the conclusion has been drawn that the carbohydrates and carbohydrate precursors of the body are in a state of dynamic equilibrium with one another through enzymatic action (1–5). Thus an added carbohydrate precursor, labeled with a carbon isotope, is greatly diluted by other carbohydrate precursors of the body. In the present study, the rôle of acetic, propionic, and butyric acids containing radioactive carboxyl carbon in glycogen formation has been investigated. In the interpretation of the data, the basic assumption is made that any substance which is in equilibrium with or is converted in the course of its metabolism into a compound known to be a carbohydrate precursor will be diluted by the metabolic pool of precursors and will hence, in part at least, become incorporated into liver glycogen if conditions favor its formation.

Although the conversion of acetate to carbohydrate precursors (6) by many of the lower forms of life has been established, there has been considerable controversy concerning the importance of this reaction in mammalian metabolism. Thunberg (7) originally suggested that succinic acid was formed by the dehydrogenation of 2 molecules of acetic acid. He suggested that this reaction occurred not only in plants but also in animal tissues. The hypothesis that acetic acid is converted to carbohydrate in mammalian tissues has received very little support from studies carried out by the usual methods. When fed as the sodium salt or as the glyceride, acetate produces no increase in glycogen in the liver over that formed by suitable controls (8, 9).

Stöhr has found that 200 mg. of sodium acetate together with 100 mg. of glucose when fed to a 100 gm. white rat result in more liver glycogen (0.2 per cent) than is present after 100 mg. of glucose only are fed. If only 100 mg. of acetate were fed with the glucose, no effect was noticed (8). In similar experiments, Stöhr (10) concluded that butyric acid likewise was converted to glycogen. Ponsford and Smedley-MacLean (11) reported that rats fed a basal diet supplemented with sodium acetate showed no significant difference in the amounts of glycogen produced in the liver when compared with the livers of rats receiving the basal diet alone.

Although Geelmuyden (12) observed an increased excretion of sugar when acetic acid was administered to a phlorhizinized dog, his results have not

been confirmed by other workers (13, 14). Cross and Holmes (15) could find no increase in carbohydrate formation from acetate by liver slices under conditions in which carbohydrate was formed from butyrate.

Although definite evidence of the conversion of butyrate to glycogen in the normal rat (9, 16) or to carbohydrate in the phlorhizinized animal is lacking (17), there is evidence that this compound may play a rôle in carbohydrate formation. Butyrate has been found to be glucogenic in perfused cat liver, D:N ratios as high as 20 being obtained (18). Under similar experimental conditions after addition of acetoacetate to the perfusing fluid, the D:N ratio remained at 5, indicating no conversion of this latter substance to carbohydrate. Blixenkrone-Møller (18) has suggested that ω oxidation of butyric acid occurs to form succinic acid, a known carbohydrate former.

Unlike acetate and butyrate, propionate does not yield ketone bodies (19) and has repeatedly been shown to form glycogen when fed to normal white rats. Eckstein (20) could find only a small increase in glycogen in the livers of white rats 4 hours after propionate was fed, but Deuel and coworkers (16) have shown that after 6 to 7 hours a considerable amount of glycogen is found in the liver. Likewise, propionate has been shown to be quantitatively converted to carbohydrate in the phlorhizinized dog (21).

In the experiments to be reported herein, acetic, propionic, and butyric acids were synthesized with the short lived radioactive isotope C^{11} in the carboxyl position. The sodium salts of these acids were fed with 400 mg. of glucose to insure adequate glycogen formation to white rats which had been fasted for 24 hours. The animals were placed in metabolism chambers and the expired carbon dioxide collected at half hour intervals for a 2 hour period. The animals were then sacrificed and the livers analyzed for their glycogen content and the radioactivity of the glycogen. From these data, conclusions have been deduced regarding the fate of the carboxyl carbon of the fatty acids with respect to carbon dioxide formation and its incorporation into liver glycogen.

EXPERIMENTAL

Synthesis of Radioactive Fatty Acids—Acetic, propionic, and butyric acids were synthesized according to the standard Grignard reaction

(1) $$RI + Mg \rightarrow RMgI$$
(2) $$RMgI + C^{11}O_2 \rightarrow RC^{11}OOMgI$$
(3) $$RC^{11}OOMgI + HOH \rightarrow RC^{11}OOH + MgI(OH)$$
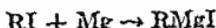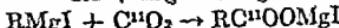
where R stands for the radicals CH_3—, C_2H_5—, and C_3H_7— respectively. Suitable yields of fatty acids based on the $C^{11}O_2$ used were obtained. In the case of acetate, actual measurements of the over-all yield demonstrated that 50 per cent of the $C^{11}O_2$ was converted to acetic acid during the synthesis.

The specific activity of C^{11} in the CO_2 was found to be equal to the specific activity of C^{11} in the fatty acids, thus indicating that Reactions 2 and 3 took place with the exclusion of other reactions.   The method of synthesis to be described below was equally applicable for all three fatty acids, acetic, propionic, and butyric.   The time required for the synthesis was approximately 45 minutes.

The radioactive carbon dioxide was obtained from the cyclotron by pumping the gas, $C^{11}O_2$, from the target chamber through 3 cc. of an alkaline solution of 2 M sodium hydroxide and $\frac{2}{3}$ M sodium carbonate.   The collecting
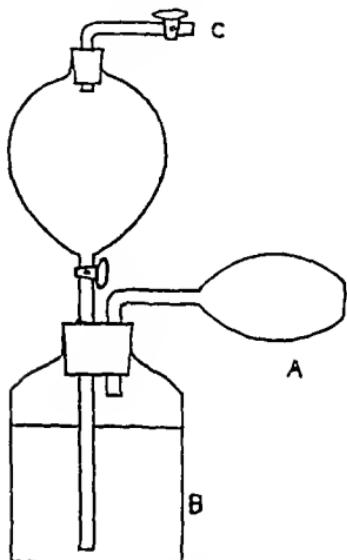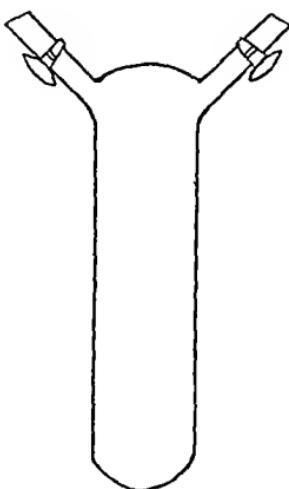
FIG. 1   FIG. 2

FIG. 1. Vessel used to collect radioactive CO_2.   A, pressure bulb; B, reservoir containing mercury; C, outlet to Van Slyke apparatus or reaction vessel.

FIG. 2. Reaction vessel used for synthesis of radioactive fatty acids.

tube was chilled in liquid air to assure efficient collection of the $C^{11}O_2$.   Upon being warmed, the alkaline solution was transferred to the reaction chamber of a Van Slyke manometric gas apparatus and the solution acidified with 4 cc. of 5 N lactic acid.   The carbon dioxide evolved was collected over mercury in a collecting vessel (Fig. 1) which was designed so that the gas could be returned to a reaction vessel (Fig. 2) containing the Grignard reagent.

The alkylmagnesium iodide compound had been previously prepared by allowing 10 mM of alkyl iodide to react with 10 mM of magnesium turnings in 10 cc. of anhydrous ether.   The reaction vessel was of about 100 cc. capacity and contained two large stop-cocks at the top.   Both stop-cocks were closed and the reaction vessel and contents immersed in liquid air.   By means of a

vacuum pump, the pressure inside the reaction vessel was reduced and the radioactive carbon dioxide was then permitted to fill the vacuum. Both stop-cocks were closed and the Grignard reagent and carbon dioxide gas were shaken for 10 minutes at approximately 0.0°. During this time, Reaction 2 had taken place. After being chilled in liquid air, the solution was then carefully acidified with 8 cc. of 2.7 N sulfuric acid. After hydrolysis of the Grignard complex had taken place, the solution was transferred to a small separatory funnel and the acid aqueous solution extracted with three 25 cc. portions of ether. The ether fractions were combined and poured into a suitable separatory funnel. 1 or 2 cc. of water were added to remove any concentrated inorganic acid from the ether. The water was separated and discarded. 1 cc. of water, containing 2 drops of 0.1 per cent phenol red, was again added and the aqueous layer made alkaline with 2 M sodium hydroxide and the fatty acid extracted from the ether layer by conversion to the sodium salt. The alkaline aqueous solution was separated and the ether solution washed with 1 cc. of water. The washings were added to the aqueous fraction. Approximately 1.5 mM of the pure fatty acid (not labeled) were added to the solution which was then well mixed. The final volume was approximately 4 to 5 cc.

An aliquot (0.3 cc.) was taken for chemical determination according to the method of Friedemann (22) and two aliquots (0.1 cc.) were taken for duplicate determinations of radioactivity. The aliquots for standard radioactivity measurements were transferred to small cups and made definitely alkaline with a drop of sodium hydroxide (approximately 1 M). This solution was allowed to evaporate to dryness. Radioactivity measurements were made in all cases by inserting these cups containing radioactive material into the interior of the ionization chamber of the electroscope. The rest of the solution of the salt of the fatty acid, to which 400 mg. of glucose had been added, was fed to a male white rat previously fasted for 24 hours. The animal was placed in the metabolism cage and the respiratory gases collected every half hour for a period of 2 hours.

At the end of the 2 hour period, the animal was sacrificed and the liver glycogen isolated as previously described (1). After the glycogen had been dried with alcohol and then with ether, it was transferred to a small cup for radioactivity measurement in the electroscope. Since, under the conditions of these experiments, the glycogen isolation was not quantitative, glycogen determinations were carried out on the sample of glycogen actually used for radioactivity measurement. The total amount of glycogen present in the liver at the conclusion of the experiment was calculated from glycogen analysis of an aliquot of the alkaline digest of the liver. The radioactivity present in the total glycogen of the liver was estimated by multiplying the radioactivity of the sample, taken for measurement, by the ratio, mg. of

total glycogen of the liver to mg. of glycogen used for radioactivity measurement.

Analyses were also made at the conclusion of the experiment on the fatty acid content of the intestines in order to estimate the amount of the fatty acid absorbed. The intestines were extracted four times with 40 cc. portions of hot water. 10 cc. of colloidal iron were added and the volume made to 200 cc. After separation of the precipitate by centrifugation, 100 cc. of solution were taken for fatty acid analysis.

Results

The results obtained after the feeding of 400 mg. of glucose with each of the three radioactive fatty acids are given in Tables I to VI inclusive. Six experiments were completed with acetate, six with propionate, and five with

TABLE I

Excretion of C^{11} in Expired CO_2 after Feeding "Carboxyl Radioactive" Acetate and 400 Mg. of Glucose

Experiment No. (1)	CO_2 expired (2)	Radioactivity in expired CO_2, per cent of amount fed					Total per cent of amount absorbed (8)
		0.0–0.5 hr. (3)	0.5–1.0 hr. (4)	1.0–1.5 hrs. (5)	1.5–2.0 hrs. (6)	Total (7)	
	m_M						
2	12.1	4.8	9.8	8.1	8.8	31.5	45.6
3	13.4	5.1	8.1	6.9	8.7	28.8	38.8
4	16.1	7.2	12.0	14.2	11.5	45.0	48.3
5*	15.2	8.4	17.2	24.4	16.6	66.6	73.0
6	13.3	5.8	9.6	13.8	12.6	41.8	85.4
7	11.0	3.1	9.9	8.6	10.5	32.1	39.2
Average...	13.2	5.2	9.9	10.3	10.4	35.8	51.5

* Omitted from average.

butyrate. Tables I, III, and V include the data on $C^{11}O_2$ excretions; Tables II, IV, and VI, the data on glycogen radioactivity. In Column 8 of Tables I, III, and V is given the radioactivity expired as carbon dioxide calculated as total per cent of the amount absorbed. The average values found are 51.5, 54.8, and 55.9 for acetate, propionate, and butyrate respectively. The radioactivity of the expired carbon dioxide calculated as per cent of the amount fed for each half hour and for the total period of time is given in Columns 3, 4, 5, 6, and 7 of Tables I, III, and V. This demonstrates that all three acids are metabolized extremely rapidly, and that after the first half hour the excretion of $C^{11}O_2$ is practically constant for the rest of the 2 hour period. The average amount of carbon dioxide expired by the animals was 13.2, 13.7, and 15.6 mM during the acetate, propionate, and butyrate experiments respectively (Column 2, Tables I, III, and V).

In order to determine whether any part of the liver glycogen carbon originated from the fatty acids, it was necessary to estimate what part of

TABLE II

Radioactivity of Liver Glycogen 2 Hours after Feeding "Carboxyl Radioactive" Acetate and 400 Mg. of Glucose

Experiment No.	Rat weight	Acetate		Liver			Glycogen radioactivity				
							Per cent of amount fed		Per cent of amount absorbed		
		Fed	Absorbed	Weight	Glycogen formed*		Total activity	Residual activity (CO₂ corrected)	Total activity	Due to CO₂	Residual activity
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	gm.	mM	mM	gm.	per cent	mg.					
2	90	2.58	1.78	3.67	2.08	72	0.53	−0.28	0.77	1.18	−0.41
3	96	3.20	2.37	4.25	1.53	60	0.76	0.20	1.03	0.76	0.27
4	130	1.75	1.63	5.74	1.05	53	1.26	0.61	1.36	0.70	0.66
5†	115	1.90	1.73	4.35	1.09	42	0.02	−0.79	0.02	0.89	−0.87
6	117	2.06	1.01	5.26	2.13	106	1.20	−0.25	2.46	2.97	−0.51
7	107	2.30	1.89	4.30	1.86	75	1.04	0.09	1.27	1.16	0.11
Average						73			1.38	1.35	0.03

* Corrected for amount found in livers of fasted controls (0.12 per cent).
† Omitted from average.

TABLE III

Excretion of C^{11} in Expired CO_2 after Feeding "Carboxyl Radioactive" Propionate and 400 Mg. of Glucose

Experiment No.	CO₂ expired	Radioactivity in expired CO₂, per cent of amount fed					Total per cent of amount absorbed
		0.0-0.5 hr.	0.5-1.0 hr.	1.0-1.5 hrs.	1.5-2.0 hrs.	Total	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mM						
1	14.2	4.5	8.2	11.7	6.7	31.1	42.0
2	13.9	2.7	4.4	5.5	6.7	19.3	42.9
3*	15.5	4.3	7.8	9.1	9.4	30.6	56.6
4	16.1	3.0	6.4	7.7	7.1	24.2	66.6
5	11.5	3.4	6.2	4.2	5.2	19.0	44.9
6	12.6	4.6	6.7	6.8	8.1	26.2	76.9
Average...	13.7	3.6	6.4	7.2	6.8	24.0	54.8

* Omitted from average.

the radioactivity of the glycogen could be accounted for by the incorporation of radioactive $C^{11}O_2$ released during metabolism of the fatty acids. This was estimated as follows:

(4) Glycogen radioactivity (% absorbed radioactivity) = radioactivity of glycogen due to $C^{11}O_2$ + radioactivity of glycogen due to other precursors containing C^{11}

where

(5) Radioactivity of glycogen due to $C^{11}O_2$ (%) =
$$\text{glycogen carbon from } CO_2 \times \text{specific radioactivity of } CO_2$$

and

(6)   Glycogen carbon from $CO_2 = 0.131 \times mM$ glycogen formed

(7) Specific radioactivity of CO_2 =
$$\frac{\text{radioactivity of expired } CO_2 \text{ (\% of absorbed radioactivity)}}{mM \text{ of total } CO_2 \text{ expired}}$$

The factor 0.131 represents the fraction of glycogen carbon derived from CO_2 determined in experiments with glucose and $NaHC^{11}O_3$ previously reported (4).

TABLE IV

Radioactivity of Liver Glycogen 2 Hours after Feeding "Carboxyl Radioactive" Propionate and 400 Mg. of Glucose

Experiment No.	Rat weight	Propionate		Liver			Glycogen radioactivity				
		Fed	Absorbed	Weight	Glycogen formed*		Per cent of amount fed		Per cent of amount absorbed		
							Total activity	Residual activity (CO₂ corrected)	Total activity	Due to CO₂	Residual activity
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	gm.	mM	mM	gm.	per cent	mg.					
1	120	1.27	0.94	4.84	1.79	81	2.40	1.62	3.24	1.05	2.19
2	146	2.23	1.00	5.31	1.08	51	0.87	0.56	1.94	0.69	1.25
3†	136	2.31	1.25	4.98	1.66	76	0.41	−0.26	0.76	1.22	−0.46
4	145	2.38	0.86	5.58	1.01	50	2.48	2.15	6.85	0.90	5.95
5	105	2.02	0.85	4.30	1.49	39	1.36	0.94	3.23	1.00	2.23
6	98	1.39	0.54	4.12	1.34	50	2.07	1.61	6.09	1.34	4.75
Average						58			4.27	1.00	3.27

* Corrected for amount in livers of fasted controls (0.12 per cent).

† Omitted from the average.

The average value of the radioactivity of glycogen due to $C^{11}O_2$ expressed as per cent of the amount of radioactivity absorbed is 1.35, 1.00, and 1.25 for the acetate, propionate, and butyrate experiments respectively (Column 11, Tables II, IV, and VI). By subtracting these values from the total amount of radioactivity incorporated after the feeding of each acid, one can estimate the amount of radioactivity in the glycogen arising from the radioactive fatty acids, exclusive of CO_2. These values, calculated as per cent of the amount absorbed are 0.03, 3.27, and 1.13 for the acetate, propionate, and butyrate experiments respectively (Column 12, Tables II, IV, and VI). From these results, it is concluded that no acetic acid is transformed directly into glycogen. As would be anticipated from data in the literature con-

cerning the ability of propionic acid to form carbohydrate, our results indicate definitely that this acid is a glycogen precursor. The results obtained after the feeding of isotopic butyrate indicate that a part, at least, of this substance is also metabolized via a carbohydrate path. However, since

TABLE V

Excretion of C^{11} in Expired CO_2 after Feeding "Carboxyl Radioactive" Butyrate and 400 Mg. of Glucose

Experiment No.	CO_2 expired	Radioactivity in expired CO_2, per cent of amount fed					Total per cent of amount absorbed
		0.0–0.5 hr.	0.5–1.0 hr.	1.0–1.5 hrs.	1.5–2.0 hrs.	Total	
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)
	mM						
1	14.9	7.2	12.2	14.6	12.3	46.3	50.8
2	14.9	7.9	13.0	12.4	10.8	44.1	55.0
3	15.8	8.8	15.4	13.2	10.3	47.7	60.2
4	16.4	8.5	16.3	12.5	7.7	45.0	60.1
5	15.8	10.0	15.0	11.2	8.8	45.0	53.4
Average...	15.6	8.5	14.4	12.8	10.0	45.6	55.9

TABLE VI

Radioactivity of Liver Glycogen 2 Hours after Feeding "Carboxyl Radioactive" Butyrate and 400 Mg. of Glucose

Experiment No.	Rat weight	Butyrate		Liver			Glycogen radioactivity				
							Per cent of amount fed		Per cent of amount absorbed		
		Fed	Absorbed	Weight	Glycogen formed*		Total activity	Residual activity (CO_2 corrected)	Total activity	Due to CO_2	Residual activity
(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)
	gm.	*mM*	*mM*	*gm.*	*per cent*	*mg.*					
1	123	1.95	1.78	5.08	2.00	96	2.15	0.85	2.36	1.43	0.93
2	120	2.01	1.60	4.59	1.37	57	1.55	0.81	1.95	0.93	1.02
3	121	1.88	1.49	4.94	1.46	66	2.02	1.15	2.54	1.10	1.44
4	110	1.67	1.25	4.73	1.74	77	2.09	1.17	2.80	1.23	1.57
5	133	1.45	1.23	6.03	1.89	107	1.89	0.56	2.24	1.57	0.67
Average..........						81			2.38	1.25	1.13

* Corrected for amount found in livers of fasted controls (0.12 per cent).

$C^{11}O_2$ appeared during the metabolism of butyrate at about the same rate as it appeared from propionate and since the amount of glycogen radioactivity after butyrate administration is considerably smaller than that after propionate administration, it may be concluded that a part of the butyric acid is oxidized by a path other than carbohydrate.

In two instances, Experiment 5, Tables I and II, and Experiment 3, Tables III and IV, the data were not included in the averages. These results were excluded from the average because they deviated quite materially from the results obtained in the experiments of the rest of the series. In both cases, the amount of radioactivity found in the liver glycogen was far below that expected on the basis of the amount of liver glycogen formed. Occasionally in control experiments, rats fasted 24 hours have been found to have excessively high levels of liver glycogen. Computation of the glycogen formed during the 2 hour experimental period would, therefore, be seriously in error if the fasting liver glycogen content had not been reduced to the average level of the animal fasted 24 hours (0.12 per cent). In such cases, it is possible then that much of the glycogen present in the liver at the end of the experiment had been initially present rather than formed during the experimental period. In the absence of appreciable glycogen formation, one would expect to find but little radioactivity in the liver glycogen isolated for radioactivity measurement. If this had occurred, results such as those recorded in the two instances cited above might be anticipated.

Isolation of Fat after Radioactive Acetate Feeding—In two experiments, liver fat was isolated both after the administration of radioactive acetate alone and after the feeding of glucose, melted butter, and radioactive acetate together. The procedure used for the fat isolation was that described by Hastings and Eichelberger (23). During the course of the 2 hour experiment, no incorporation of acetate into liver fat could be detected. Although there was no evidence of the conversion of acetate to liver fat during this experimental period, the possibility that this reaction could occur in an experiment of longer duration is not excluded. It is of interest, in this connection, that although Bloch and Rittenberg (24) did not find deuterium in the fatty acids of animals fed deuterioacetate, they point out the possibility that the carbon of deuterioacetic acid might be used in fatty acid synthesis even though the deuterium is not. Although no evidence is available for the utilization of acetate in the synthesis of naturally occurring fatty acids, Bloch and Rittenberg have demonstrated that acetate is involved in cholesterol formation. Propionate and butyrate are not converted to cholesterol, however.

DISCUSSION

In Fig. 3, a comparison is made between the results obtained after the feeding of glucose plus carboxyl-labeled fatty acid and those obtained after *dl*-carboxyl-labeled lactate was fed. The darkened areas of the four columns represent in each case the fraction of the total liver glycogen radioactivity which is accounted for by the C^{11} entering the glycogen as (+4) carbon. Although there was considerable variation from experiment to experiment in the amount of radioactive carbon dioxide incorporated

into liver glycogen after glucose, lactate, and pyruvate feeding, and radio-active bicarbonate injection, the average values of each of these three series were quite consistent. Thus, after lactate, glucose, and pyruvate feeding, 11.4, 13.1, and 14.2 per cent of the glycogen carbon formed respectively had its origin from (+4) carbon. Because of the close agreement of these values, it was felt that an approximate correction accounting for the C^{11} entering the glycogen as $C^{11}O_2$ could be applied to the radioactivity values found in liver glycogen after the feeding of radioactive fatty acids. After the feeding of radioactive carboxyl-tagged *dl*-lactate, it was found that only
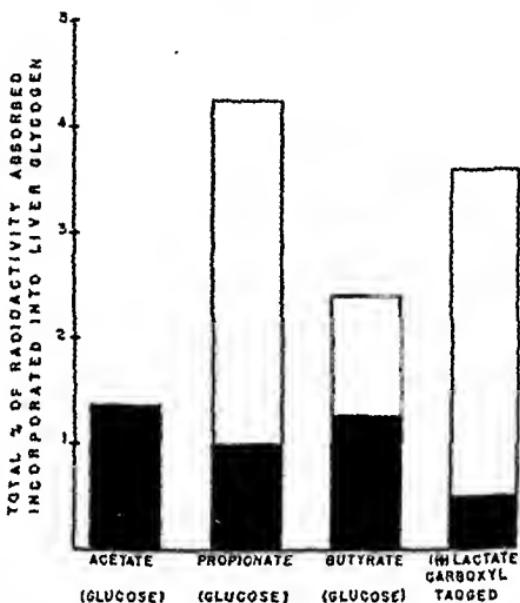
FIG. 3. Comparison of radioactivity present in newly formed liver glycogen following administration of glucose plus carboxyl-labeled acetate, propionate, and butyrate. Results from labeled lactate experiments are included at the right. The heights of the columns indicate total radioactivity present. The shaded areas indicate radioactivity of glycogen due to incorporation of $C^{11}O_2$ which was derived from the metabolism of the acids.

12 per cent of the total activity of the glycogen formed resulted from the $C^{11}O_2$ produced during the metabolism of the radioactive lactic acid (3). In the case of the fatty acids, however, the correction due to radioactive carbon dioxide accounted for a greater percentage of the glycogen radioactivity. 23, 55, and 98 per cent of the total radioactivity of the liver glycogen after propionate, butyrate, and acetate feeding respectively was accounted for by the $C^{11}O_2$ incorporation. These results are interpreted as indicating that propionate and butyrate may be metabolized via carbohydrate pathways, but that acetate is not.

In these experiments, the intracellular specific radioactivity of (+4) carbon was estimated from the specific radioactivity of carbon in the expired carbon dioxide. The assumption is also made that the specific activity of radioactive (+4) carbon is the same throughout the tissue fluids of the body at any given moment. This assumption is basic for the calculation of the radioactivity present in glycogen due to (+4) carbon after radioactive organic acid administration. In other words, it is assumed that the intracellular and extracellular specific activity of (+4) carbon is essentially the same whether the (+4) carbon was produced intracellularly, as in the combustion of the fatty acids, or introduced into the body extracellularly as after intraperitoneal injection of radioactive bicarbonate. That this assumption is probably justified is indicated by the experiments of Ball, Tucker, Solomon, and Vennesland (25). They found that, soon after intravenous injection of radioactive bicarbonate, the specific activity of C^{11} in the serum is equal to the specific activity of C^{11} in the pancreatic juice, a secretion of intracellular origin, thus indicating rapid equilibrium of both intracellular and extracellular (+4) carbon.

Since, as shown by Cori and Cori (26), the unnatural isomer $d(-)$-lactate is not appreciably converted to glycogen, the values of the carboxyl dl-lactate in Fig. 3 have been expressed in terms of the $l(+)$-lactate absorbed, assuming that $d(-)$-lactate and $l(+)$-lactate are absorbed at equal rates (26). Consequently, the average radioactivity values of the experiments with the dl-lactic acid have been multiplied by 2. On this basis, 3.6 per cent of the $l(+)$-carboxyl lactate absorbed was converted to glycogen. It is appreciated that the results obtained after fatty acid plus glucose feeding and those after dl-lactate feeding without glucose are not strictly comparable. The amount of radioactivity incorporated into liver glycogen after a radioactive organic acid is fed is probably directly proportional to the amount of liver glycogen formed and inversely proportional to the size of the carbohydrate metabolic pool. Although the effective carbohydrate pool is increased by simultaneous feeding of glucose with the radioactive fatty acids, considerably more glycogen was formed in these experiments than in the lactate experiments. For these reasons, it is probably fortuitous that the values for the net radioactivity of the glycogen formed after the feeding of radioactive dl-lactate (calculated as $l(+)$-lactate) and propionate agree so closely.

The use of glucose to stimulate glycogen formation in the presence of labeled fatty acids occurred to us following the experiments of Moss and Schoenheimer (27) who found that considerable quantities of deuteriotyrosine were formed from deuteriophenylalanine when large amounts of normal non-isotopic tyrosine were included in the diet in addition to the isotopic phenylalanine. Likewise, Stetten and Schoenheimer (28) have shown that

in rats palmitic acid is converted into palmitoleic acid and stearic acid, although these substances were supplied in the butter fat of the stock diet. These experiments lend support to the view that the reactions concerned with the function and metabolism of protein and fat are in dynamic equilibrium. A comparable point of view is adopted in the present paper in which the reaction of fatty acids to glycogen formation from glucose is studied.

SUMMARY

1. Three carboxyl radioactive fatty acids, acetic, propionic, and butyric, containing radioactive carboxyl carbon have been synthesized and fed with glucose to fasted white rats.

2. Evidence is presented which indicates that propionic and butyric acids are converted to liver glycogen, but that acetate is not.

3. Approximately 50 per cent of the radioactive fatty acid absorbed was excreted in the respiratory gases as carbon dioxide over a 2 hour period.

4. After radioactive acetate was fed with and without other substances, no C^{11} could be detected in the liver fat fraction 2 hours after administration.

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THE ELECTROPHORETIC PROPERTIES OF GLOBIN FROM VARIOUS SPECIES

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Recently, Reiner, Moore, Lang, and Green (1) reported on the electrophoretic analysis of bovine globin in glycine-hydrochloric acid buffers at pH 2.6 and 3.7. They found that under these conditions bovine globin separated into two components. They also found that the mobilities and proportion of these two components were only slightly affected by the method of purification. Globin preparations containing large amounts of denatured globin had essentially the same properties as purified globin.

At the time this paper appeared we were investigating the electrophoretic properties of human globin, using phosphate buffers over a pH range of 5.2 to 7.9. Under these conditions we had not found any evidence of separation into two components, but in view of the report of Reiner *et al.* decided to extend our studies to include the pH value of 2.7, and both bovine and rabbit globin.

EXPERIMENTAL

Material and Methods

Globin was prepared according to the method of Anson and Mirsky (2); human globin from red cells remaining after removal of plasma from outdated bank blood, bovine and rabbit globin from red cells obtained after centrifugation of fresh oxalated blood. The native globin was usually freed from denatured globin by dissolving it in water, neutralizing by dialysis against $M/30$ dipotassium phosphate, and precipitating the rest of the denatured globin by bringing the solution to 40 per cent saturation with ammonium sulfate. The native globin was then precipitated by bringing the filtrate to 70 per cent saturation with ammonium sulfate. The precipitate was centrifuged down, dissolved in the buffer, and dialyzed against the particular buffer under investigation. Usually four dialyses against 500 cc. of the buffer were sufficient to remove the ammonium sulfate, and this was followed by dialysis against the final lot of 2000 cc. used for electrophoresis.

The phosphate buffers used for electrophoresis had an ionic strength of 0.1 and ranged in pH from 5.2 to 7.9. In the electrophoresis at pH 2.7, the buffer described by Reiner *et al.* (1), containing 0.1 M glycine-hydrochloric acid and 0.12 M sodium chloride, was used. Since the ionic strength

of this buffer differs from that of the phosphate buffers, the results in the two buffers are not comparable.

The electrophoretic analyses were made by means of the Tiselius apparatus (3) with the Toepler schlieren optical arrangement as modified by Longsworth (4). Electrophoresis was allowed to proceed for 2 to 5 hours, 4.5 to 6.5 volts per cm. being used.

TABLE I
Mobility of Globin in Phosphate Buffers of Ionic Strength 0.1

Species	pH	Mobilities*	Species	pH	Mobilities*
		$\mu \times 10^5$			$\mu \times 10^5$
Human	5.2	+4.0	Human	7.6	−0.1
"	5.5	+3.1	"	7.9	−1.3
"	6.2	+2.0	Rabbit	5.5	+3.1
"	6.8	+0.7	Bovine	5.6	+2.7
"	7.3	+0.1			

* Calculated on the basis of the descending boundaries.

FIG. 1. Longsworth patterns of human globin in phosphate buffer, $\mu = 0.1$, at pH 5.5. Left, ascending pattern; right, descending pattern.

Results

The data obtained by electrophoresis of globin from the three species in phosphate buffers are given in Table I. Over a range of pH 5.2 to 7.9, human globin migrates as a single component with an isoelectric point at pH 7.5. The electrophoretic diagram for human globin at pH 5.5 is shown in Fig. 1. Bovine and rabbit globin were studied in phosphate buffer only at pH 5.5. It was found that at this pH they also migrated as a single component, with a mobility identical with that of human globin.

In the glycine-hydrochloric acid buffer of pH 2.7, we found, as did Reiner *et al.*, that bovine globin separates into two components. The mobilities which we obtained, however, differed markedly from those reported by

these authors (see Table II). It appeared possible that the difference in mobilities might be due to the method of preparation of the native globin. Accordingly, the effect of modifications in the method of preparation on the mobilities was investigated: (a) the globin was neutralized with sodium hydroxide and precipitated by ammonium sulfate as described by Anson and Mirsky (2), then dissolved in, and dialyzed against, distilled water

TABLE II

Mobility of Globin in Glycine-Hydrochloric Acid Buffer of pH 2.6

Species	Method of purification	Mobilities*	
		Fast component	Slow component
		$\mu \times 10^5$	$\mu \times 10^5$
Bovine	K_2HPO_4 and $(NH_4)_2SO_4$	6.8	4.8
"	NaOH and $(NH_4)_2SO_4$	6.7	4.6
"	No purification	6.6	4.5
Rabbit	K_2HPO_4 and $(NH_4)_2SO_4$	6.9	4.9
Human	" " "	5.8	4.1
Bovine	Data of Reiner *et al.* (1)	9.4	6.6

* Calculated on the basis of the descending boundaries.
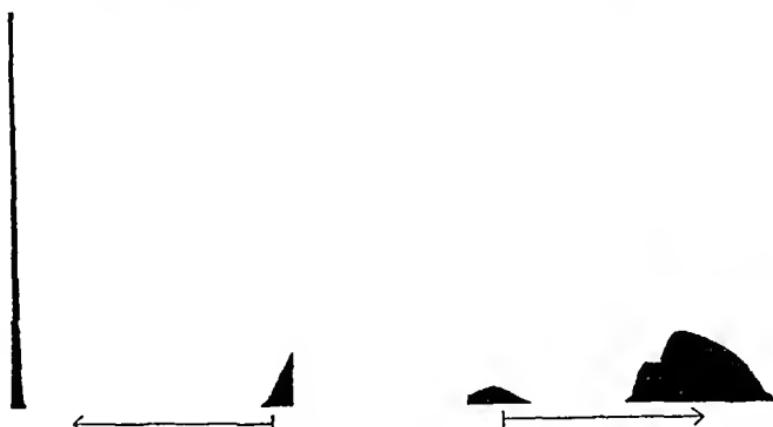
FIG. 2. Longsworth patterns of human globin in glycine-hydrochloric acid buffer, 0.1 M, containing 0.12 M sodium chloride, at pH 2.7. Left, ascending pattern; right, descending pattern.

until free of ammonium sulfate, and then dialyzed against the buffer, and (b) crude globin was dissolved in water and dialyzed against the buffer without purification. In each case, however, the same mobilities were obtained for the two components (see Table II). Storage of the crude globin in the dry state does not appear to influence the mobility, since identical values were obtained after the globin had been kept in a vacuum desiccator for a period of 3 months.

Rabbit globin when subjected to electrophoresis in glycine-hydrochloric acid buffer at pH 2.7 has properties similar to those of bovine globin. Two distinct components were found with mobilities the same as those we had found for bovine globin (Table II).

Human globin, however, differs from bovine and rabbit globin in this respect. There is some evidence of separation of the globin; see Fig. 2 which shows the pattern obtained after electrophoresis for 290 minutes with 4.9 volts per cm. The break in the curve indicates that in human globin there are two components, but that there is not a great difference in their relative mobilities. These were calculated to be 5.8×10^{-5} and 4.1×10^{-5} sq. cm. per volt per second for the fast and slow component respectively, but the values must be considered as only approximate.

DISCUSSION

There is an interesting similarity between our data for globin and those found by other investigators for plasma albumin. Thus Luetscher (5) found that both human and horse serum albumin separated into two components when subjected to electrophoresis at pH 4.0. He also found a difference in the mobilities of the fast and slow components in the two species studied. Cohn (6) states that at pH 7.7 albumins from human, bovine, or horse sera migrate as single components with almost identical mobilities, and Tiselius and Kabat (7) obtained similar results for horse, pig, rabbit, and monkey albumin.

Similarly we found that at pH 5.5, rabbit, bovine, and human globin migrated as a single component with the same mobility. At pH 2.7, however, there was separation into two components whose mobilities were the same for bovine and rabbit globin, but differed for human globin. This difference between bovine and human globin becomes even greater if our values for human globin are compared with those found by Reiner et al. (1) for bovine globin.

We are unable to give any explanation for the variation between our data for bovine globin at pH 2.7 and those found by Reiner et al. (1). These authors found, as we did, that neither the method of preparation of native globin nor the presence of denatured globin had any marked effect on the proportions of the two components or on their respective mobilities. The possibility exists that variations in the preparation of the crude globin itself have been responsible for the differences found in the two laboratories.

SUMMARY

1. In phosphate buffer at pH 5.5, globin prepared from human, bovine, and rabbit hemoglobin migrates as a single component with identical mobilities for the three species.

2. In phosphate buffers over a range of pH 5.2 to 7.9, human globin migrates as a single component with an isoelectric point of pH 7.5.

3. In glycine-hydrochloric acid buffer at pH 2.7, bovine and rabbit globins separate into two distinct components with identical mobilities for the two species. Human globin also separates into two components, but the separation is not so marked, nor are the mobilities the same as those found for bovine and rabbit globin.

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THE ABSORPTION SPECTRUM OF LUCIFERIN AND OXIDIZED LUCIFERIN

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The ostracod crustacean, *Cypridina hilgendorfii*, has a gland in the head region which manufactures and stores two chemical substances, an enzyme, luciferase, and a substrate, luciferin, whose interaction in the presence of water and oxygen gives rise to a luminescence. These compounds remain stable for years if the animals are stored in a dry state. It is necessary only to grind up the dried material and extract with suitable solvents to obtain luciferin or luciferase for quantitative studies of the luminescent reaction *in vitro* (Harvey (8)).

It has been shown (Chase (4)) that the visible absorption spectrum of solutions of partially purified *Cypridina* luciferin undergoes a definite series of changes during exposure to air, the rate increasing with increasing pH. These measurements were made with Shlaer's spectrophotometer (Shlaer (10)). While this instrument has a very high precision, its operation is rather slow and it is consequently not possible to measure the complete visible absorption spectrum of a solution whose color is unstable. However, spectra containing four or five points can be obtained if measurements are made as rapidly as possible at four or five selected wave-lengths in succession and are repeated until the color of the solution has become stable. A curve relating photometric density ($\log I_0/I$) to time is then plotted for each of the wave-lengths measured and from these curves skeleton absorption spectra can be drawn by interpolation. These indicate the color of the solution at any given instant after the onset of the reaction. From absorption spectra that were measured by this method it was apparent that during oxidation of luciferin solutions (without luciferase) the absorption maximum —originally in the violet region of the spectrum—shifts rapidly to about 480 mμ, and then gradually disappears, without any further qualitative change, to give finally an almost colorless solution.

Since the rate of disappearance of luciferin (as measured by the total light obtainable on addition of luciferase) approximately paralleled the rate of change of the absorption spectrum of the luciferin solutions at various pH values, it was assumed that the labile color was a property of the luciferin itself. The initial shift of maximum to 480 mμ was interpreted as representing the reversible oxidation of luciferin (Anderson (3)), and the subsequent disappearance of this maximum as representing a secondary irreversible reaction, probably also an oxidation.

433

The measurements described above have been repeated, and a number of additional ones made, with the Hardy recording spectrophotometer (6) at the Massachusetts Institute of Technology.   This instrument can trace a complete visible absorption spectrum upon graph paper in from 2 to 4 minutes.   Consequently, the details of the spectrum can be measured and the position of maxima well established, even though the color of the solution is not stable.   The effect of pH on the course of the color changes was studied in order to get absorption curves with greater detail than those reported in the earlier paper.   The absorption spectrum of a luciferin solution free of oxygen was also measured to determine whether or not the color change could occur in the absence of oxygen.   In addition, luciferin extractions that had been carried through one and two cycles of purification were studied and the amount of labile color was compared with the concentration of luciferin in the two solutions.   Finally, the absorption spectrum was measured during the luminescent reaction itself.   This is possible with the Hardy spectrophotometer because it does not respond to continuous, but only to intermittent light, and the measurements are therefore not affected by luminescence in the absorption cell.   The primary purpose of these four sets of experiments was to determine whether the color changes were actually characteristic of the dissolved luciferin itself or merely related to some other component of the solution.

Materials and Methods

The luciferin was extracted from dried, ground *Cypridina* organisms and was purified by the method of Anderson (2).   Some extractions were used which had been carried through only one cycle of purification; others had been carried through a second cycle.   The latter are visibly less colored than the former.   The luciferase was freed of practically all colored impurities and of a certain amount of protein impurity by dialysis against distilled water.   As the pH falls during prolonged dialysis, precipitation of considerable inactive material occurs and the practically colorless active supernatant can then be used as a luciferase stock solution (Giese and Chase (5)).

The pH of the experimental solutions was adjusted with 0.2 M phosphate buffers (disodium and monopotassium phosphates).

In all of the experiments, luciferin that had been dried by removing the butyl alcohol solvent *in vacuo* was used as the starting material.   This dried residue was then dissolved in a small amount of 0.1 N hydrochloric acid, phosphate buffer was added, and finally an amount of 0.1 N sodium hydroxide exactly equivalent to the acid was run in, and the solution was transferred to an absorption cell.   pH values were checked with the glass electrode.

In those experiments in which anaerobic conditions were not maintained,

measurement of the absorption spectrum was begun immediately after the free acid had been neutralized by the sodium hydroxide. Since the procedures and dilutions necessarily differed considerably in the different types of experiments, they will be described in greater detail in the following section.

EXPERIMENTAL

Effect of Hydrogen Ion Concentration on Absorption Spectrum and Changes Which It Undergoes—Two aqueous solutions of doubly purified luciferin, treated as described above and buffered at pH 6.8 and 5.1, respectively,
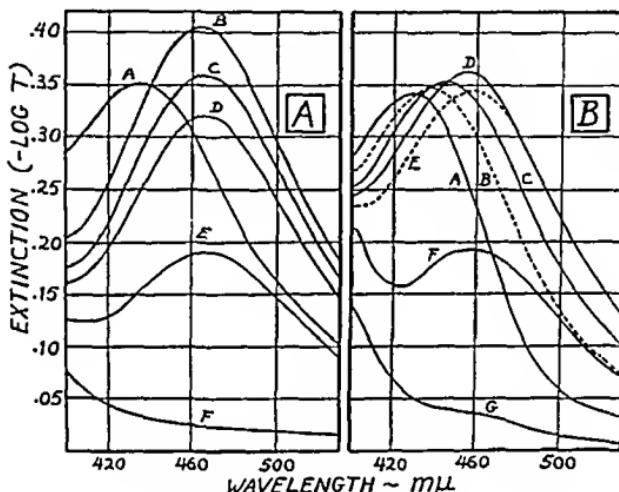
Fig. 1. *A* shows the changes which occur in the visible absorption spectrum of a luciferin solution of pH 6.8 during exposure to air. Curve A is the spectrum 5 minutes after the luciferin was dissolved. Curve B was measured after a total elapsed time of 19 minutes, Curve C after 31 minutes, Curve D after 44 minutes, Curve E after 81 minutes; Curve F is the spectrum after 2 days. *B* shows similar measurements on a luciferin solution of pH 5.1. Curve A was measured 4 minutes after the luciferin was dissolved, Curve B after 16 minutes, Curve C after 30 minutes, Curve D after 59 minutes, Curve E after 81 minutes, Curve F after 300 minutes, and Curve G after 26 hours.

were measured with the Hardy recording spectrophotometer at intervals during exposure to air in an absorption cell whose optical depth was 10 mm. Fig. 1 shows absorption spectra[1] of these two solutions. Several facts are at once evident from these curves. First, the absorption spectrum of the freshly dissolved luciferin is very similar in the two solutions, although

[1] In all the graphs, extinction (negative logarithm₁₀ of the transmission measured by the spectrophotometer) has been plotted on the ordinate against wave-length in mμ on the abscissa.

their pH is different. Second, the absorption maximum shifts from a value of about 430 mμ to one of about 460 or 470 mμ in both cases, and this change is much faster at pH 6.8 than at pH 5.1. Third, in both cases the absorption maximum at about 460 to 470 mμ subsequently disappears at a relatively slow rate until the solution approaches a colorless condition. However, in the case of the solution at pH 5.1, a product is evidently formed which behaves as an acid-base indicator with a stronger blue-violet absorption. This substance is apparently produced during the second step in the color change; that is, during the slow disappearance of the absorption maximum at 460 to 470 mμ. Preliminary measurements in the visible and ultraviolet regions with the Harrison and Bentley rapid recording spectrophotometer (Harrison and Bentley (7)) show that an increase of absorption in the region between 320 and 400 mμ is correlated with the decrease at 470 mμ (see Fig. 6).

Dependence of Color Changes upon Oxygen—Luciferin solutions remain stable practically indefinitely, so far as potential luminescence is concerned, in absence of dissolved oxygen. Therefore, if the changes in absorption spectrum of luciferin solutions represent luciferin, they should not occur if oxygen is not present. The apparatus designed for this experiment is shown diagrammatically in Fig. 2. A fused glass absorption cell of 50 cc. capacity and 50 mm. optical depth was equipped with a cover of thick Pyrex glass through which two holes had been bored. A glass tube projected through one of these holes to just below the lower surface of the cover. This tube was fitted with a stop-cock (*A*) of sufficient bore to permit entrance of a thin glass tube from a burette, so that a measured volume of solution could be run into the cell. Through the other hole in the Pyrex plate a glass tube ran to the bottom of the absorption cell in such a way as not to obstruct the optical path through the cell. This tube connected through a stop-cock (*B*) with the lower end of a glass cylinder of 40 cc. capacity. At the upper end of this cylinder was a 3-way stop-cock (*C*).

6 cc. of butyl alcohol solution of luciferin were transferred from the anaerobic storage flask into the absorption cell through the bore of stop-cock *A* by means of the modified burette. The whole apparatus was then put into a vacuum desiccator and the butyl alcohol was removed with a pump, leaving a thin film of dry material (largely luciferin) on the bottom of the absorption cell. The apparatus was removed from the desiccator and 18 cc. of 0.2 M phosphate buffer of pH 6.75 and 10.5 cc. of 0.100 N sodium hydroxide were put into the glass cylinder through the bore of stop-cock *C*, and one arm of *C* was then connected with the purified hydrogen line with lead tubing and de Khotinsky cement. The free openings of stop-cocks *C* and *D* were connected with water traps by rubber tubing.

Through the bore of stop-cock *A* were now run 10.5 cc. of 0.100 N hydro-

chloric acid to dissolve the luciferin in the absorption cell. Oxidation of luciferin occurs very slowly in 0.1 N hydrochloric acid (Anderson (2)), and so no effort was made to free the acid solution of air before introducing it. A and D were immediately connected together with lead tubing and de Khotinsky cement and all the stop-cocks were turned so as to allow puri-
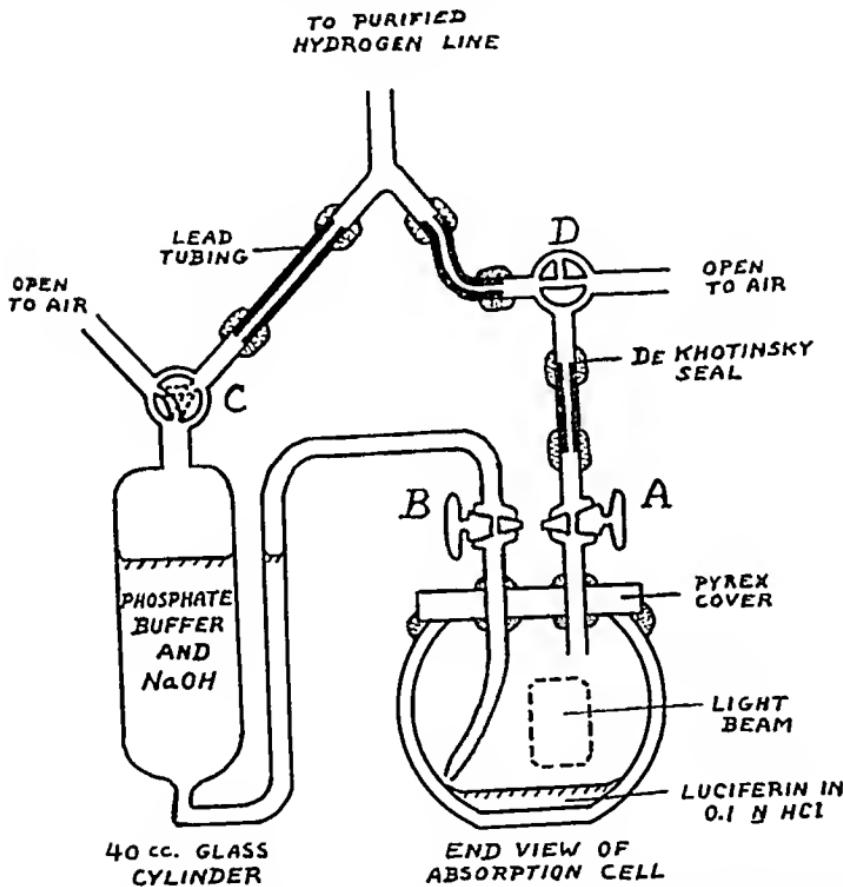
Fig. 2. Diagrammatic plan of the apparatus used to prepare an anaerobic solution of luciferin of pH 6.8 in an absorption cell, for measurement of the absorption spectrum in the absence of air. For details of the procedure see the text.

fied hydrogen to flow into the absorption cell, over the surface of the acid-luciferin solution, through B, up through the solution in the cylinder, and out of the apparatus to the air through the open arm of C. The hydrogen flowed in this direction at a slow rate for 9 hours, ample time to deaerate both solutions.

Stop-cocks A and C were now turned so as to close off completely the absorption cell and the cylinder. The part of the tubing between the

hydrogen line and C was flushed out with hydrogen, D having been closed. Stop-cock C was next turned so as to connect the cylinder and the hydrogen line, and A and D were immediately opened to the air, with the result that the solution in the cylinder was forced over into the absorption cell and mixed with the acid-luciferin solution, thereby producing a pH of approximately 6.8 in the final mixture.

The hydrogen was allowed to bubble through the mixed solutions in the absorption cell for 2 hours to insure complete deaeration, and stop-cocks A and B were then closed and the absorption cell and cylinder disconnected from the hydrogen line and transported to the spectrophotometer. Absorption spectra of this solution are shown in Fig. 3. The luciferin used
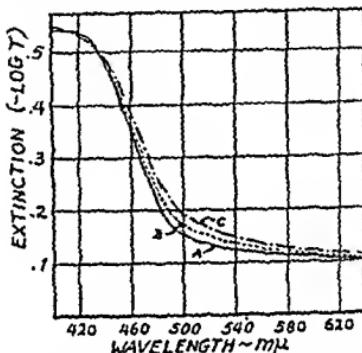
FIG. 3. Absorption spectra of a hydrogen-saturated solution of luciferin that has been carried through a single cycle of purification. Four spectra measured during the course of an hour fell exactly on Curve A. Curve B was measured after air had been passed through the solution for 1 minute, and Curve C after the solution had been exposed to air for 60 minutes. The color change does not occur in the absence of oxygen.

in the measurements had been carried through only one cycle of purification and the curves consequently do not have the shape of those measured on doubly purified luciferin (see, for example, Fig. 1), because a greater amount of stable yellow color, which would have been removed by the second cycle of purification, was still present. The form of the absorption spectrum is immaterial in this experiment, however, since its purpose is to demonstrate the complete stability of the color of the solution in absence of air. Six absorption spectra were measured, four during an hour in which the absorption cell remained closed and therefore free of oxygen, and two following the admission of air to the cell. The first four curves were identical and all fell exactly on Curve A in Fig. 3. Curve B represents the color 10 minutes after the stop-cocks of the absorption cell had been opened and air passed slowly through the solution for 1 minute. The absorption of the

solution has increased at wave-lengths longer than about 440 mμ. Curve C represents the solution after passive exposure to air for a period of 60 minutes. The absorption has continued to increase at wave-lengths longer than 440 mμ and has decreased slightly at shorter wave-lengths. This experiment demonstrates conclusively that the changes in the absorption spectrum of luciferin solutions are dependent on oxygen, just as is the loss of luciferin from such a solution when the luciferin content is determined by measuring the total luminescence. This indicates an identity between luciferin and the substance responsible for the changes in the absorption spectrum, although it does not prove the point conclusively.

Relation between Labile Color of Luciferin Solutions and Luciferin Concentration As Measured by Total Light Emission—The fact that the velocity of the change in the absorption spectrum of a purified luciferin solution increases with increase in pH in a manner that is qualitatively similar to the rate of disappearance of luciferin, as measured by total light content (Chase (4)), indicates a relationship between the luciferin and the changes in the absorption spectrum. Furthermore, as described above, neither the changes in the absorption spectrum nor the loss of luciferin (measured by total light content) occurs in absence of dissolved oxygen. Even more convincing evidence that the change in absorption spectrum represents luciferin itself can be obtained by comparing the difference in labile color between two luciferin solutions that have been carried, respectively, through one and two cycles of purification (the method of Anderson (2)) with the difference in luciferin content of these two solutions as measured by their total luminescence. If the luciferin is responsible for the labile color, there should be a proportionality between the latter and total luminescence.

As a measure of labile color, as contrasted with total color (labile plus stable color), the curve of photometric density (log I_0/I) against wavelength of a fully oxidized solution of luciferin was subtracted from that of a solution in which luciferin had been freshly dissolved. In Fig. 4 seven absorption spectra are shown. Curve A is from an aqueous solution (pH 6.8) of doubly purified luciferin measured 5 minutes after the luciferin was dissolved.[2] Curve B is from the same solution after the color had become stable. Curve C is the difference between Curve A and Curve B, and can be taken as representing the labile color of the solution. Curve D

[2] The butyl alcohol was removed *in vacuo* from 7 cc. of butyl alcohol solution of luciferin that had been carried through two cycles of purification. The dry residue was dissolved in 1.00 cc. of 0.100 N hydrochloric acid; 2.00 cc. of 0.20 M phosphate buffer of pH 6.8 were added and then 1.00 cc. of 0.100 N sodium hydroxide to neutralize the free acid. The optical depth of the absorption cell was 10 mm.

is the absorption spectrum of a singly purified luciferin solution,[3] measured 15 minutes after the luciferin and accompanying soluble impurities were dissolved. Exploratory measurements had shown that the changes in the absorption spectrum were slower in the case of the singly purified luciferin solution than in the doubly purified one and it was for this reason that the 15 minute curve was used instead of the 5 minute curve. Curve E represents the final, stable color of the singly purified luciferin solution. Curve F was obtained by subtracting Curve E from Curve D, and is taken as representing the labile color of the solution of singly purified luciferin.
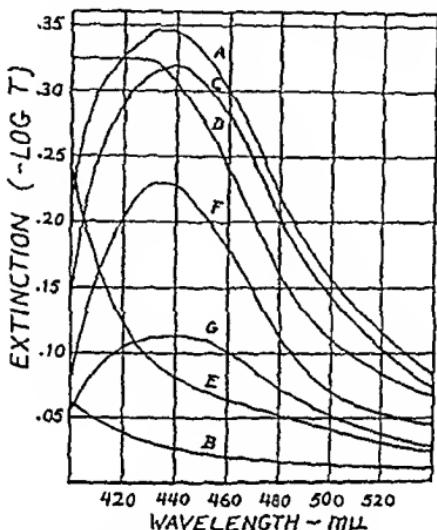
FIG. 4. Curves F and G are calculated absorption spectra representing the labile color in two luciferin solutions of different degrees of purity. Curves A, B, D, and E represent the data used in computing Curves F and G by the method described in the text. Curve C gives the original values from which those of Curve G were calculated to correct for concentration differences between the two luciferin solutions.

From the data contained in foot-notes 2 and 3 the dilution factor by which Curve C must be multiplied to make it directly comparable with Curve F can be calculated. This factor turns out to be 0.357 and Curve G represents the values of Curve C multiplied by it. The heights of Curves F and G at $\lambda = 435$ mμ were taken as measures of the labile color in the singly and doubly purified luciferin solutions, respectively. These values are 0.230 and 0.116 and their ratio is 1.98. The total luminescence from

[3] The butyl alcohol was removed *in vacuo* from 5 cc. of luciferin solution that had been carried through a single cycle of purification. The dry residue was dissolved in 1.00 cc. of 0.100 N hydrochloric acid; 4.00 cc. of 0.20 M phosphate buffer of pH 6.8 were added and then 1.00 cc. of 0.100 N sodium hydroxide. The optical depth of the absorption cell was 10 mm.

samples of the singly and doubly purified luciferin solutions was measured
with the light-integrating apparatus described by Anderson (1). It was
found that 1.00 cc. of the solution of singly purified luciferin had a total
luminescence represented by 130.0 volts, while 1.00 cc. of the solution of
doubly purified luciferin gave a total luminescence of 77.7 volts.[4] The
ratio of these two values is 1.69. This figure differs by only 15 per cent from
the ratio of the labile color in the two solutions.

The rather good correspondence between these two ratios indicates that
the labile color of luciferin solutions, and hence the changes in absorption
spectrum which such·solutions undergo, is a property of the luciferin mole-
cule itself and not of impurities extracted with it from the *Cypridina*
organisms.

Absorption Spectrum of Luciferin Solution during Luminescent Reaction—
As was mentioned earlier, the design of the Hardy spectrophotometer is
such that it permits the measurement of absorption spectra of colored solu-
tions in which continuous light emission is occurring. An experiment was
therefore designed to determine whether the changes in the absorption
spectrum of purified luciferin solutions during oxidation by air are dupli-
cated during the relatively much more rapid oxidation of luciferin which
accompanies luminescence in the presence of the enzyme, luciferase.

The residue from 10 cc. of butyl alcohol solution of luciferin (carried
through two cycles of purification) was dissolved in 2.0 cc. of 0.10 N hydro-
chloric acid, and 2 cc. of 0.2 M phosphate buffer, pH 6.8, were added, fol-
lowed by 2.0 cc. of 0.10 N sodium hydroxide. 2.5 cc. of this mixture were
immediately put into each of two identical absorption cells (10 mm. optical
depth). 1.5 cc. of distilled water were added to the solution in one cell
and 1.5 cc. of a strong luciferase solution to the other. A brilliant blue
luminescence occurred in the latter cell and the absorption spectrum was
measured at once.

Immediately after the first curve had been traced for the luminescent
solution, the other solution which lacked luciferase was measured. The
two solutions were measured alternately for an hour. They were then left
for 8 hours and remeasured. The solution which had no luciferase was
measured once more after a total of 20 hours had elapsed. Finally the
absorption spectrum of a solution of luciferase of exactly the same con-
centration as was used in the luminescent sample was measured. On the
assumption that the luciferase undergoes no change in color during the
luminescent reaction, its color could be subtracted from that of the luciferin-
luciferase mixture and should allow an exact comparison between the
velocity and character of the color changes in the two solutions.

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[4] The light emission is measured in arbitrary units, expressed as volts required to
balance the total output of the photocell in the apparatus (Anderson (1)).

In Fig. 5, A are shown absorption spectra of the luciferin solution which did not contain luciferase and which was consequently undergoing slow, spontaneous, non-luminescent oxidation. In Fig. 5, B are the absorption spectra which were measured on the solution to which luciferase had been added. These curves have been corrected for the slight color of the luciferase itself.
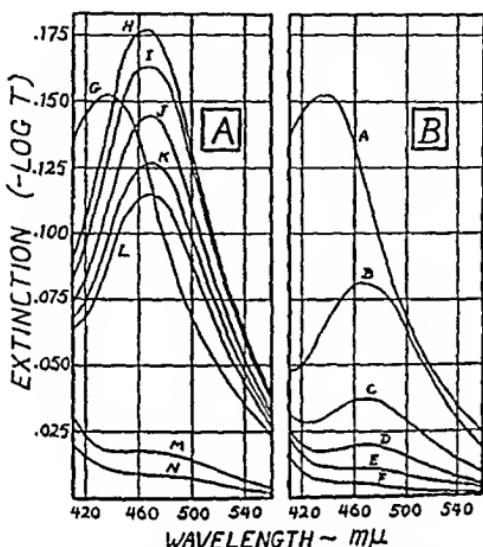
FIG. 5. The curves shown in A are absorption spectra of a luciferin solution measured during exposure to air. Curve G is the spectrum immediately after the luciferin was dissolved, Curve H after 9 minutes, Curve I after 20 minutes, Curve J after 30 minutes, Curve K after 39 minutes, Curve L after 49 minutes, Curve M after 455 minutes, and Curve N after 1140 minutes. In B are shown absorption spectra of a luciferin solution in which luminescence is occurring due to the action of the enzyme, luciferase. Curve A is the spectrum immediately after the luciferin was dissolved, Curve B is measured 1 minute after luciferase was added, Curve C after 12 minutes, Curve D after 22 minutes, Curve E after 40 minutes, and Curve F after 457 minutes. The changes in the absorption spectrum are about 100 times as rapid during luminescent oxidation by luciferase as during non-luminescent oxidation.

Inspection of Fig. 5 shows two things: (1) Essentially the same changes take place in the absorption spectrum of the two solutions and (2) they occur about 100 times as fast when luciferase is present as when it is absent. Curve A in Fig. 5, B and Curve G in Fig. 5, A were obtained from identical luciferin solutions which were measured as soon as possible after the luciferin had been dissolved. The original color of the solution for both samples is therefore represented by these curves. It is evident that during the luminescent reaction between luciferin and luciferase the same shift in absorption maximum from about 430 mμ to about 470 mμ occurs which is

observed during the spontaneous, non-luminescent oxidation of luciferin. Moreover, this change must be exceedingly rapid during the luminescent reaction.   These results are additional evidence that the changes in absorption spectrum are attributable to the luciferin itself, rather than to impurities.

DISCUSSION

From the results of the experiments that have been described in the preceding section, it seems certain that the luciferin molecule in aqueous solution possesses a definite absorption band in the visible region of the spectrum and that during both luminescent and non-luminescent oxidation it is converted to a compound or compounds having a different visible absorp-
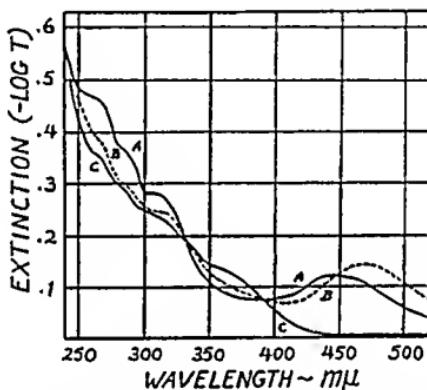
Fig. 6. Visible and ultraviolet absorption spectra measured with the Harrison and Bentley spectrophotometer.   Curve A was measured as soon as possible after the luciferin was dissolved.   Curve B was measured after exposure of the solution to air for 32 minutes, and Curve C after 24 hours.

tion band.   The fact that the changes in the absorption spectrum are the same whether luminescence be initiated by adding luciferase or whether the luciferin be allowed to oxidize spontaneously in air need not necessarily mean that the reaction products are identical in the two cases.   If the luciferase reacts with a different group on the luciferin molecule than is affected in the spontaneous, non-luminescent oxidation, the changes in the absorption spectrum might still be identical provided the light-absorbing group itself were not radically affected.   However, the simpler interpretation would be that the products of the luminescent and non-luminescent reaction are identical, and this would imply that the luciferin molecule is altered in the same way in both reactions.   The careful experiments of Anderson (3) indicate that the product of the luminescent reaction is different from that of the non-luminescent oxidation, since he finds the latter

reversible and the former not. Although the absorption spectrum measurements are superficially not in accord with his results, they do not actually contradict them, for the reason that has been given above.

Preliminary measurements of the visible and ultraviolet absorption spectrum of *Cypridina* luciferin with the Harrison and Bentley spectrophotometer (7) show, in addition to the visible band, ultraviolet bands at about 310 to 320 mμ, and at 280 and 260 mμ, with complete absorption below 240 mμ. This spectrum, shown in Curve A of Fig. 6, bears a striking resemblance to the spectra of certain naphthoquinone and anthraquinone derivatives measured by Morton and Earlam (9). However, it has been found that the ultraviolet absorption spectrum of luciferin, like the visible spectrum, is unstable in the presence of air. Measurements with the rapid recording spectrophotometer of Harrison and Bentley show that during the first stages of non-luminescent oxidation the absorption decreases in the region from 250 to 320 mμ, and subsequently increases the region from 320 to 400 mμ, the latter change taking place during the time that the visible band at about 465 mμ is disappearing (see Fig. 6). Careful measurements of the ultraviolet spectrum of hydrogen-saturated luciferin solutions should be valuable in the determination of the structure of the molecule if the luciferin can be obtained sufficiently free of impurities for reliable ultraviolet studies.

SUMMARY

By means of the Hardy recording spectrophotometer the visible absorption spectrum of solutions of purified luciferin was measured during spontaneous non-luminescent oxidation at pH 5.1 and 6.8. In each case an initial absorption maximum at about 435 mμ is replaced by one at about 465 mμ which subsequently disappears leaving an almost colorless solution. The velocity of these changes is greater at pH 6.8 than at pH 5.1. At the latter pH the final, stable solution absorbs more strongly in the blue-violet region, indicating that a compound may be formed which has the properties of an acid-base indicator. Measurements with the Harrison-Bentley recording spectrophotometer show that changes also occur in the ultraviolet absorption spectrum of luciferin solutions during exposure to air.

The changes in the visible absorption spectrum of luciferin solutions do not occur when the solution is saturated with hydrogen to the exclusion of oxygen.

The labile color of solutions of luciferin is directly proportional to the concentration of luciferin determined by total luminescence measurements.

The same sequence of changes in the absorption spectrum of luciferin solutions occurs when luminescence is initiated by addition of the enzyme, luciferase. In this case, however, the velocity is about 100 times as great as when the enzyme is not present.

It is concluded from these data that luciferin itself, and not accompanying impurities, is responsible for the observed changes in the visible absorption spectrum during exposure to air.

Although measurements of the ultraviolet absorption spectrum of partially purified luciferin solutions suffer from instability of the spectrum in the presence of dissolved oxygen and from possible presence of impurities, maxima of absorption appear to exist at about 310 to 320 mμ, 280, and 260 mμ, with complete absorption below 240 mμ.

I wish to thank Dr. E. Newton Harvey for his interest and helpful criticism during the course of this work.

Through the kindness of Dean John W. M. Bunker, space and facilities were provided in the Biology Department at the Massachusetts Institute of Technology while the measurements were being made with the Hardy and the Harrison and Bentley spectrophotometers. I am extremely grateful to all these men and to the members of the Biology Department staff for the privileges and aid which were accorded me.

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THE MECHANISM OF COZYMASE SYNTHESIS IN THE HUMAN ERYTHROCYTE: A COMPARISON OF THE RÔLES OF NICOTINIC ACID AND NICOTINAMIDE

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It is now well established that the administration of adequate amounts of nicotinic acid results in a large increase in the V factor (coenzymes I and II) content of human erythrocytes (1–3). This synthesis has been observed *in vitro*, and is independent of the leucocytes (4). Recently, Hoagland and Ward (3) have reported that administration of comparable quantities of nicotinamide are without effect on the erythrocyte V factor concentration. The present report is concerned with an attempt to determine the basis for this difference and thereby gain some insight into the mechanism of cozymase synthesis.

EXPERIMENTAL

Methods

The V factor estimations of Hoagland and Ward depended upon the reduction of nitrate to nitrite by *Hemophilus influenzae* with subsequent colorimetric determination of the nitrite (3). It was thought of value to repeat their experiments with the turbidimetric assay of the growth of *Hemophilus parainfluenzae* described by Kohn and Bernheim (5). The accuracy of this procedure with pure solutions of cozymase has been checked by simultaneous comparison with the yeast fermentation method (6) and has been further confirmed by the excellent agreement obtained between the values for cozymase in human and dog erythrocytes (7) and rat and dog kidney cortex (6, 8). The results of the V factor assays were calculated as if all the V factor were present as diphosphopyridine nucleotide and are expressed as micrograms of diphosphopyridine nucleotide per cc. of erythrocytes. By total nicotinic acid is meant the nicotinic acid indicated by the colorimetric analysis and it is expressed as micrograms per cc. of red cells. Bound nicotinic acid represents the nicotinic acid present in the pyridine nucleotides and is calculated from the V factor assay. Unbound nicotinic acid is the arithmetical difference between total and bound nicotinic acid in the erythrocytes. Plasma nicotinic acid cannot be further subdivided and is obtained directly from the colorimetric assay.

Effects of Nicotinamide and Nicotinic Acid on Erythrocyte V Factor Concentration in Vivo—Nicotinamide (3 gm.) was given during 3 days in divided doses (four per day) to a number of healthy young males. 3 hours after the last dose, blood was drawn by antecubital venepuncture and analyzed for V factor and for plasma and cell nicotinic acid. 24 hours later the same subjects were started on a similar course of 3 gm. of nicotinic acid in divided doses over 3 days. Blood samples drawn 3 hours after the last dose were assayed in like fashion. While the time intervals were considerably smaller than those reported by Hoagland and Ward, they have proved satisfactory in our experience (5). Typical results obtained on two of the subjects are shown in Table I.

TABLE I

Effect of Nicotinic Acid and Nicotinamide Administration on Erythrocyte V Factor Concentration

	Subject H. I. K.					Subject P. H.				
Time	Cell diphos-phopy-ridine nucleo-tide	Cell nicotinic acid		Plasma nico-tinic acid	Remarks	Cell diphos-phopy-ridine nucleo-tide	Cell nicotinic acid		Plasma nico-tinic acid	Remarks
		Bound	Total				Bound	Total		
days	*γ per cc.*	*γ per cc.*	*γ per cc.*	*γ per cc.*		*γ per cc.*	*γ per cc.*	*γ per cc.*	*γ per cc.*	
0	72	13.2	14.0	0.6		74	13.6	16.1	0.5	
4	103	19.0	24.1	4.0	After amide	101	18.6	19.2	2.1	After amide
8	201	37.2	38.7	2.4	" acid	194	36.0	46.4	6.3	" acid

While nicotinamide did not produce the dramatic effects on erythrocyte V factor concentration seen with nicotinic acid, it was not without effect. Nicotinamide feeding consistently raised the erythrocyte V factor content by 25 to 40 per cent under these conditions, while like amounts of nicotinic acid produced increases which varied from 90 to 300 per cent in a similar period. While it cannot be stated with certainty that the increase in V factor activity was due solely to an increase in the pyridine nucleotides, this would seem to be indicated by the excellent agreement between total and bound nicotinic acid. Since nicotinamide nucleoside is but one-third as active in the V factor assay as an equimolecular amount of cozymase, the presence of appreciable quantities of nicotinamide nucleoside would result in a considerable discrepancy between total and bound nicotinic acid. However, this experiment does not preclude the possibility of nicotinamide nucleoside as an intermediary in cozymase synthesis.

Permeability of Nicotinic Acid and of Nicotinamide in Vivo—Under the conditions employed above, only small increases in plasma nicotinic acid were observed. The chemical method used did not permit differentiation

between nicotinic acid and nicotinamide in either cells or plasma. Since poor synthesis of V factor from nicotinamide might be due merely to impermeability of the red cell membrane to nicotinamide, the following experiment was undertaken. A single oral dose of 1 gm. of nicotinamide was taken by each of two healthy adult males and of 500 mg. of nicotinic acid by two others. Blood samples were taken for analysis at frequent intervals. The results are given in Tables II and III.

TABLE II

Plasma and Erythrocyte Analysis after Ingestion of 1 Gm. of Nicotinamide

Time	Nicotinic acid, subject H. K.				Nicotinic acid, subject H. P. S.			
	Bound	Total	Unbound	Plasma	Bound	Total	Unbound	Plasma
hrs.	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*
0	15.1	16.1	1.0	<1	14.8	16.2	1.4	<1
0.5	15.1	30.1	15.0	15.3	14.8	43.0	28.2	26
1.0	15.1	41.0	25.9	23.6	14.8	39.0	24.2	28
1.5	15.1	42.0	26.9	25.7	14.8	35.0	20.2	22
2.0	15.1	28.6	13.5	16.4	14.8	30.5	15.7	14.9
4.0	15.1	21.7	6.6	7.6	14.8	22.2	7.4	7.3
6.0	15.1	18.3	3.2	3.7	14.8	18.3	3.5	3.5

TABLE III

Plasma and Erythrocyte Analysis after Ingestion of 0.5 Gm. of Nicotinic Acid

Time	Nicotinic acid, subject H. I. K.				Nicotinic acid, subject P. H.			
	Bound	Total	Unbound	Plasma	Bound	Total	Unbound	Plasma
hrs.	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*	γ *per cc.*
0	15	15.0	0	1	14.7	15.0	0.3	1
1	15	16.7	1.7	1.3	14.7	22.0	7.3	7.6
2	15	17.3	1.3	2.1	14.7	21.0	6.3	5.9
4	15	19.6	4.6	1.9	14.7	19.1	4.4	1.4
6	15	18.3	3.3	1.6	14.7	16.7	2.0	1.1

Tables II and III show that both nicotinic acid and nicotinamide are rapidly absorbed from the alimentary canal. Maximum plasma concentrations were observed after 60 minutes. The administration of these single large doses had no effect on cell V factor concentration over the period measured. The excellent agreement between the unbound nicotinic acid of the cells and the nicotinic acid of the plasma is apparent. This parallel rise and fall of the concentrations of both substances in the cells and plasma indicates that the erythrocytes are freely permeable to both. This being so, the different behavior of these two substances in promoting

the synthesis of cozymase must be attributed to specific chemical or enzymatic factors.

V Factor Synthesis by Human Erythrocytes in Vitro—Synthesis of V factor from nicotinic acid by the human erythrocyte *in vitro* has been reported by Kohn and Klein (4). However, such synthesis from nicotinamide has also been reported (2). It was thought of interest to compare the rates of these syntheses.

In each instance 1.0 cc. of freshly drawn blood was incubated for 24 hours at 35° with 0.1 cc. of 0.9 per cent NaCl containing 250 γ of nicotinic acid or its amide. The controls were incubated with saline alone. Sterile precautions were observed throughout the procedure. 0.2 cc. of this mixture was then taken for V factor analysis. The results are shown in Table IV. Each value represents the mean of three independent analyses.

TABLE IV

V Factor Synthesis by Human Erythrocytes in Vitro

The V factor is calculated as if it were all coenzyme I.

Substrate, 0.1 cc. 0.9 per cent NaCl containing	Cell V factor
	γ per cc.
No addition	77
Nicotinic acid, 250 γ	153
Nicotinamide, 250 γ	97
Nicotinic acid, 250 γ, + nicotinamide, 2000 γ	161

In these experiments nicotinamide produced an increase of 30 to 50 per cent in the V factor content of the erythrocytes. This must be taken to indicate that human red blood cells do possess the ability to synthesize cozymase from nicotinamide. However, it is possible that the nicotinamide must first be hydrolyzed to nicotinic acid within the cell, since the latter compound produced increases of 80 to 140 per cent in the erythrocyte V factor concentration. The presence of nicotinamide did not inhibit synthesis from nicotinic acid but rather, if anything, appeared to enhance this synthesis.

Decomposition of Cozymase after Hemolysis—It has been established that the decomposition of cozymase by animal tissues (brain, liver, kidney cortex, muscle) *in vitro* is accomplished by cleavage of the nucleosidic linkage between nicotinamide and the remainder of the cozymase molecule (9, 10). It seemed of interest in the present connection to determine whether the same mechanism operates in the human erythrocyte.

To this end, aliquots from the same samples of blood were allowed to stand for 30 minutes in 4 times their volume of saline, water, 1 per cent nicotinamide, and 1 per cent nicotinic acid (pH 7.4). Samples were then

taken for V factor analysis. The results are collected in Table V. Each value is the mean determined with three blood samples.

It is apparent that the destruction of cozymase following hemolysis is inhibited by nicotinamide but not by nicotinic acid. Since this has also been found to be true of the enzyme system of rat tissues, it would appear that the pathway of destruction of cozymase is the same in both instances.

This conclusion was supported by the finding that the enzymatic decomposition of cozymase was not accompanied by hydrolysis of the nicotinamide moiety. Human erythrocytes were hemolyzed in water and the proteins precipitated by tungstic acid in the manner employed for total nicotinic acid analysis (7). One aliquot was then taken for analysis in the usual fashion. A second was treated with sodium hypobromite (11) to convert any nicotinamide present to β-aminopyridine which gives virtually no color in the Koenig reaction when metol (p-methylaminophenol

TABLE V

Nicotinamide Inhibition of Decomposition of Cozymase after Hemolysis
The V factor is calculated as if it were all coenzyme I.

Medium	Cell V factor
	γ per cc.
0.9% NaCl...	84
Water.........................	4
Nicotinic acid, 1%................ .... · ··········	3
Nicotinamide, 1%...	80

sulfate) is used as the coupling agent. This sample was then analyzed for nicotinic acid. In four such determinations chromogenic material equivalent to but 1 γ of nicotinic acid per cc. of erythrocytes was obtained. Of the total of 16 γ of nicotinic acid determined by the usual technique, after hemolysis and enzymatic decomposition of cozymase, 15 γ must, therefore, be nicotinamide. It is noteworthy that the amount of nicotinic acid thus determined is of the same order of magnitude as the usual difference between total cell nicotinic acid and that calculated from the V factor estimation. To complete this description of the mechanism of cozymase decomposition after hemolysis it would be essential to establish whether or not inorganic phosphate is liberated in this process. However, this did not seem feasible in the presence of the relatively large amounts of phosphate ordinarily present in the medium after hemolysis.

DISCUSSION

The foregoing data definitely indicate that cozymase synthesis within the human erythrocyte does not proceed merely by reversing the pathway

of decomposition. The enzyme system which, *in vitro*, destroys cozymase functions by the liberation of nicotinamide, not nicotinic acid, from the remainder of the cozymase molecule. Yet nicotinamide is, at best, but one-third as potent as nicotinic acid in promoting cozymase synthesis by human erythrocytes *in vivo* or *in vitro*. Moreover, nicotinamide fails to inhibit cozymase synthesis from nicotinic acid, while it does inhibit the enzymatic decomposition of cozymase. Since inhibitors have usually been found to inhibit biologically reversible reactions in both directions (12), this is further evidence of the difference between the mechanisms of synthesis and decomposition of cozymase by the human erythrocyte.

SUMMARY

1. Simultaneous nicotinic acid and V factor analysis of human erythrocytes after the ingestion of nicotinic acid or its amide, and after *in vitro* incubation, indicates that the amide is, at best, one-third as potent as the acid in stimulating cozymase synthesis.

2. The human erythrocyte is freely permeable to both nicotinic acid and nicotinamide.

3. The enzymatic decomposition of cozymase after hemolysis appears to proceed in the same manner observed with rat tissues, *in vitro*.

4. It is concluded that the synthesis of cozymase by the human erythrocyte is not accomplished by reversal of the events in decomposition.

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THE DETERMINATION OF ASCORBIC ACID IN WHOLE BLOOD AND ITS CONSTITUENTS BY MEANS OF METHYLENE BLUE; MACRO- AND MICROMETHODS*

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In 1934 Martini and Bonsignore (1) developed a photochemical method for the determination of ascorbic acid, using methylene blue in the presence of strong light as the oxidant. They found that the reaction was most valid at pH 4 to 4.5. In 1936 Lund and Lieck (2) reported a similar method. Both these procedures involved simultaneous irradiation and titration. In 1938 Guthe and Nygaard (3) studied the course of this reaction by means of a photelgraph, and observed that the wave-length of light effective in activating the reaction corresponded to the absorption of methylene blue and was 560 to 680 mμ. Their reaction mixture was pH 2.7. This method eliminated visual titration, the progress of oxidation and decoloration being recorded by a photelgraphic tracing.

The photosensitized reaction of methylene blue and ascorbic acid at the optimal reaction pH 4.5 is rapidly reversible. When the light is removed, the leucomethylene blue changes back to the colored reduced form at such velocity as to make photoelectric colorimetry impractical. In a preliminary report from this laboratory (4) of the method amplified in the procedures reported here, this difficulty was overcome by changing the pH of the reaction mixture at completion of the activated reduction of the methylene blue to an acidity that retards the reverse oxidation. Thus illumination could be discontinued and photoelectric colorimetry conveniently carried out without change of color.

Because methylene blue under the condition of the procedures developed is a more sensitive and specific oxidation-reduction indicator for ascorbic acid than are the commonly used indophenol indicators, its use provides micromethods that are more satisfactory than those heretofore available (5–7).

Apparatus—Special centrifuge hematocrit tubes for separating the buffy layer of whole oxalated blood, as described by Butler and Cushman (4). The separation of the buffy layer in these tubes depends upon the selection of a tube whose proportions are such that the meniscus of the red cells and the buffy layer of the centrifuged whole blood in the tube will come within

the constricted stem. These tubes can be obtained from the Macalaster-Bicknell Company, Cambridge, Massachusetts, in sets of four which cover a hematocrit range of 15 to 55 per cent. They are centrifuged in centrifuge cups 150 mm. × 35 mm.

Tubes for CO saturation and HPO_3 precipitation. A heavy walled, Pyrex Thunberg tube, 120 × 20 mm., with a round bottom and a ground glass joint at the top into which fits the ground glass end of the top overhang bulb. The small hole in the ground glass portion of the top is so placed in relation to the overhang bulb that the side arm opens into the tube when the overhang bulb is directly opposite the side arm. The side arm is 25 mm. long, with an internal diameter of approximately 3 mm. The assembled Thunberg tube has an over-all length of approximately 136 mm. and may be centrifuged in a 50 cc. centrifuge cup, with the side arm just clearing the rim of the cup. The tube is so placed in the centrifuge cup that the side arm and bulb will face down while centrifuging. The tube is held in place in the cup by a thin ring of cork or rubber surrounding it and fitting snugly into the cup. A rubber band or "ty-on" is twisted about the bulb and side arm to immobilize the two parts during centrifugation.

A 2-way stop-cock with three thick walled rubber tubings for connection to the side arm of the Thunberg tube, water vacuum pump, and CO reservoir. By turning the cock the saturation tube can be alternately evacuated and filled with CO. A manometer is inserted between the stop-cock and reaction tube.

Photoelectric colorimeter, Evelyn,[1] Filter 660, with transmission from 635 to 720 mμ.

Activating light. A 500 watt electric light with reflectors focused to illuminate the colorimeter tube placed in a beaker of water.

Reagents—

All reagents should be prepared with glass-distilled water and all reagent vessels should be rinsed and all cells laked with such water.

Oxalate anticoagulant solution. 3 gm. of ammonium oxalate and 2 gm. potassium oxalate per 100 cc. of distilled water (8). The required number of cc. of this solution for the sample of blood to be used, on the basis of 0.2 cc. per 5 cc. of blood, is measured into a 25 cc. Erlenmeyer flask and evaporated to dryness with a current of air.

0.5 M acetic acid. 3 cc. of glacial acetic acid plus distilled water to 100 cc.

0.25 M sodium acetate buffer solution. 3.4 gm. of $NaC_2H_3O_2 \cdot 3H_2O$ and distilled water to 100 cc. plus 0.2 cc. of 0.5 M acetic acid. The pH should be 7. Test with brom-thymol blue and add more acid if necessary. Preserve with a few drops of toluene.

Methylene blue (methylthionine chloride, Merk's reagent grade) solution. 10 mg. of methylene blue plus distilled water to 100 cc.; keeps at least 2 weeks in the refrigerator.

Buffered dye solutions. A, macroanalysis: 1.2 mg. per cent of methylene blue in 0.125 M acetate buffer solution. This is made fresh daily by mixing 3 cc. of 10 mg. per cent methylene blue, 12.5 cc. of 0.25 M sodium acetate buffer, and 9.5 cc. of distilled water. B, microanalysis: 0.5 mg. per cent of methylene blue in 0.1 M acetate buffer solution made fresh daily by mixing 1 cc. of 10 mg. per cent methylene blue, 8 cc. of 0.25 M acetate buffer, and 11 cc. of distilled water. The addition of the buffered dye solution to the HPO_3 filtrate and blank solution as specified in the procedures should bring the pH of the final solution to between 4.3 and 4.5.

HPO_3 solutions. 0.5, 25, 30, 40, and 55 per cent solutions. These solutions should be stored in an ice box and made up fresh at least every 2 weeks.

Hydrochloric acid solutions. 0.1 N and 0.3 N.

CO gas. This is prepared and stored according to the directions of Peters and Van Slyke (9).

Procedure

Preparation of Samples of Plasma, White Cells Plus Platelets, and Whole Blood—About 15 cc. of venous blood are collected in the chemically clean flask containing 0.6 cc. of the oxalate anticoagulant solution previously dried. If one anticipates any difficulty in getting the blood sample, a drop of 20 per cent potassium oxalate should be put in the syringe. The slightest clotting will prevent the clear separation of the white cell layer from red cells and thus will invalidate the results.

Determine the hematocrit on 0.5 cc. of this blood. According to hematocrit reading select the appropriate special centrifuge hematocrit tube, and to it transfer by means of a capillary pipette the amount of blood between 10 and 12 cc. needed to bring the white cell meniscus, after centrifuging, within the constricted stem of the tube. Cover with tin-foil. After the tube is centrifuged half an hour at high speed, the plasma is poured off and used for analysis of plasma ascorbic acid. This plasma keeps in the refrigerator about 2 hours unchanged (10, 11). The tube is centrifuged again at high speed for 2 hours to complete separation of the buffy layer of white cell platelets from the red cells. The plasma is removed as completely as possible by means of a capillary pipette.

While this centrifugation is taking place, the whole blood and plasma analyses can be completed.

Whole Blood Analysis on 0.5 Cc. Sample—To 7.3 cc. of glass-distilled water in the Thunberg tube add 0.5 cc. of blood and 1 drop of caprylic alcohol.

The laked blood samples can be kept for many hours at room temperature or for as much as 24 hours in a refrigerator without reduction of the ascorbic acid. Place 0.2 cc. of 40 per cent HPO_3 in the overhang cup. Connect the two parts, using a small amount of Lubriseal to make the joint air-tight and the apposition such as to connect the side arm with the tube. Connect the side arm to the thick walled rubber tubing of the 2-way stop-cock leading alternately to the vacuum or the CO reservoir. Connect in such a way that the tube is at an angle of about 60° with the overhang bulb dependent. In this position the possibility of the acid splashing over into the laked blood during the evacuation is minimized. Evacuate the Thunberg tube for 1 minute and then fill with CO for 1 minute, shaking gently throughout the entire procedure. Repeat this alternate evacuation and filling with CO three times. With the tube filled with CO, turn the overhang cup to close the Thunberg tube. Disconnect the side arm from the 2-way stop-cock. Turn the overhang cup still further, so that it is directly above the side arm, and secure in this position by slipping a rubber band or "ty-on" over the neck of the overhang cup and around the tube. Mix the contents of the overhang cup and tube, drain to the tube, and centrifuge at medium speed for 3 minutes. After centrifuging, the precipitate should be bright red. The supernatant fluid is rapidly filtered through a No. 43 Whatman filter paper to remove any specks which may be floating on the surface.

To 4 cc. of the filtrate in a colorimeter tube add 1 cc. of the buffered dye Solution A for macroanalysis. Mix, place the tube in a beaker of cool tap water, and illuminate with activating light for exactly 1 minute. Rapidly add 5 cc. of 0.1 N HCl delivered by blowing through a pipette with a large tip. Immediately discontinue the illumination, mix the contents by inverting the tube, and place the tube in a colorimeter having Filter 660 and a 10 cc. aperture in a bakelite holder. The galvanometer reading is taken at once with the center setting[2] adjusted to read 100 with the dye completely decolorized.

For the blank 1 cc. of the same buffered dye solution is added to 4 cc. of 0.5 per cent HPO_3 in a colorimeter tube and treated as the blood filtrate above.

$$Calculation— \frac{(\text{Log } Gs - \log Gb)K \times 100}{0.25 \times 1000} = \text{mg. \% ascorbic acid}$$

where Gs is the galvanometer reading of the unknown filtrate, Gb is the reading of the dye-blank solution, and K is the constant of proportionality as given in Table I.

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[2] When the galvanometer reading is adjusted to 100 with a tube containing completely reduced dye filtrate solution in place, the removal of the tube results in a galvanometer reading called the center setting.

Whole Blood Analysis on 0.2 Cc. Sample—To 5 cc. of glass-distilled water in the Thunberg tube deliver 0.2 cc. of capillary blood collected by skin prick in a Folin capillary pipette. Add 1 drop of caprylic alcohol. Place 0.1 cc. of 40 per cent HPO_3 in the overhang cup, connect the apparatus and evacuate, saturate with CO, and centrifuge as described above. Using a 4 cc. pipette with a tight cotton filter tip, withdraw slightly more than 4 cc. of the supernatant to the pipette, remove the cotton tip, adjust the meniscus to the 4 cc. mark, and deliver to the colorimeter tube. Add 1 cc. of buffered dye Solution B for microanalysis and illuminate as directed above for 1 minute. Then rapidly add 1 cc. of 0.3 N HCl and discontinue the illumination. The colorimetric analysis of unknown and blank is carried out as described except that the 6 cc. aperture in the bakelite holder is used.

$$Calculation— \frac{(\text{Log } Gs - \log Gb)K \times 100}{0.151 \times 1000} = \text{mg. \% ascorbic acid}$$

Whole Blood Analysis on 1 Cc. Sample of Blood Low in Ascorbic Acid Content—This procedure is limited to blood containing no more than 0.4 mg. per cent of ascorbic acid. Within this limit, however, it provides conditions under which concentrations of 0.0, 0.1, 0.2, and 0.3 mg. per cent can be clearly distinguished and thus for blood of low concentration provides greater accuracy than the foregoing procedures.

To 6.8 cc. of glass-distilled water in the Thunberg tube add 1 cc. of blood and 1 drop of caprylic alcohol. Place 0.2 cc. of 55 per cent HPO_3 in the overhang cup. Proceed with saturation, precipitation, filtration, and colorimetric assay as directed for the analysis of the 0.2 cc. sample.

$$Calculation— \frac{(\text{Log } Gs - \log Gb)K \times 100}{0.5 \times 1000} = \text{mg. \% ascorbic acid}$$

Plasma Analysis—The plasma proteins are precipitated from 0.5 cc. of plasma in 7.3 cc. of glass-distilled water by the addition of 0.2 cc. of 30 per cent HPO_3. 4 cc. of filtrate are treated with Solution A as in the procedure for the corresponding amount of whole blood.

$$Calculation— \frac{(\text{Log } Gs - \log Gb)K \times 100}{0.25 \times 1000} = \text{mg. \% ascorbic acid}$$

If plasma is hemolyzed so that the acid precipitation would result in oxidation of ascorbic acid, the 0.5 cc. of plasma is added to 7.3 cc. of water in a Thunberg tube and the analysis carried on as directed in the analysis of 0.5 cc. of whole blood.

When the ascorbic acid content is low, the accuracy of the analysis may be increased by modifying the procedure as follows: The protein is precipitated from 1 cc. of plasma in 6.8 cc. of water by 0.2 cc. of 40 per cent

HPO₃. 4 cc. of filtrate are treated as directed in the analysis of 0.2 cc. of whole blood.

$$Calculation— \frac{(\text{Log } Gs - \log Gb)K \times 100}{0.5 \times 1000} = \text{mg. \% ascorbic acid}$$

White Cell Platelet or Buffer Layer Analysis—After centrifuging the cells for 2 hours at high speed, remove as much plasma as possible by means of a capillary pipette. With a similar pipette transfer approximately 10 to 12 mg. of the buffy layer of white cell platelets to a Pyrex centrifuge tube containing 0.5 cc. of glass-distilled water, which has been weighed with the water before the addition of the sample of buffy layer. The difference in the two weights gives the mg. of sample analyzed. The cells are laked and pulverized with a glass rod which is then rinsed with 4.5 cc. of glass-distilled water. If the analysis cannot be completed, the ascorbic acid will keep in this state 24 hours in the ice box. To precipitate the protein add 0.1 cc. of 25 per cent HPO₃. Shake the contents thoroughly and filter through a small filter. 4 cc. of the supernatant fluid are transferred to a colorimeter tube and treated as the filtrate in the analysis of 0.5 cc. of whole blood.

Calculation—

$$\frac{(\text{Log } Gs - \log Gb)K \times 100}{\text{Mg. white cell platelet in 4 cc. fluid analyzed} \times 1000}$$

$$= \text{mg. ascorbic acid per 100 gm. white cell platelets}$$

Results

The data of Table I show the variation in the constant of proportionality, as the concentration of methylene blue varies, and thus the error observed in the analyses of solutions containing known amounts of ascorbic acid. For the analyses at the 10 cc. volume the range of 0.5 to 10.0 γ of ascorbic acid analyzed corresponds to from 0.2 to 4.0 mg. of ascorbic acid per 100 cc. of blood or plasma when procedures requiring 0.5 cc. samples are used for analysis. Thus the methylene blue concentration of 1.2 mg. per 100 cc. will provide a buffered dye solution suitable for the analysis of blood or plasma containing from 0.2 to 2.0 mg. of ascorbic acid per 100 cc. with an error probably no greater than ±4 per cent. For the analyses at the 6 cc. volume the range from 0.25 to 5.0 γ of ascorbic acid analyzed corresponds to from 0.17 to 3.3 mg. of ascorbic acid per 100 cc. of blood or plasma when procedures requiring 0.2 cc. samples are used for analysis. A buffered dye solution containing 0.5 mg. per cent of methylene blue will, therefore, permit the analysis of samples containing from 0.2 to 1.3 mg. per cent of ascorbic acid with an error no greater than ±5 per cent. If 1 cc. of blood or plasma is used for analyses at 6 cc. volume, this concentration of buffered

dye solution will permit the analysis of samples containing from 0.05 to 0.4 mg. per cent of ascorbic acid with an error no greater than ± 2 per cent. Hence, as stated above, the procedure for 1 cc. samples of whole blood

TABLE I

Determination of Error of Colorimetric Procedure As Shown by Variation in Proportionality Constant, K^*, in Analysis of Known Amounts of Ascorbic Acid When Buffered Dye Solutions of Different Methylene Blue Concentrations Are Used

Volume analyzed	Ascorbic acid analyzed	Methylene blue† per 100 cc.							
		0.5 mg.		1.2 mg.		1.6 mg.		2.0 mg.	
		Galvanometer	K	Galvanometer	K	Galvanometer	K	Galvanometer	K
cc.	γ								
10	Blank	69.0		55.5		44.0		34.5	
10	0.5	72.5	24						
10	1.0	75.5	26	60.5	24				
10	1.5	78.8	26						
10	2.0	83.0	25	67.0	25				
10	2.5	88.0	24	71.3	24	55.8	25	42.8	26
10	5.0			88.0	25	71.0	24	54.0	26
10	7.5					87.0	25	70.0	25
10	10.0							85.0	26
		0.5 mg.		0.67 mg.		1.0 mg.			
6	Blank	60.3		52.0		41.0			
6	0.25								
6	0.50	65.0	15	56.0	16	44.0	16		
6	0.75								
6	1.00	70.3	15	60.5	15	47.0	17		
6	1.50	77.0	14			50.3	17		
6	2.00	83.0	14	70.8	15	55.0	16		
6	2.50					58.5	16		
6	5.00					80.0	17		

* Where $K = \dfrac{\text{micrograms ascorbic acid}}{(\log Gs - \log Gb)}$ and Gs is galvanometer reading of dye-ascorbic acid solution and Gb is galvanometer reading of dye-blank solution.

† The concentration of methylene blue in 1 cc. of buffered dye solution used in the analysis.

should be used for the accurate analysis of blood containing under 0.4 mg. of ascorbic acid per 100 cc.

The observations of Martini and Bonsignore (1) concerning the range of pH within which the reaction is optimal have been verified. Within the pH range of 4.0 to 4.7 the proportionality factors K for the procedures

described have been found to have a constancy within the limits of the experimental error of the method.   At pH below 4 the velocity of the reac-

TABLE II

Comparison of Macro- and Microanalyses of Venous Whole Blood and Microanalyses of Simultaneously Obtained Capillary Whole Blood and Recovery by Macromethod of Ascorbic Acid Added to Whole Blood

The results are expressed in mg. per 100 cc.

Subject	Venous, micro-analysis	Capillary, microanalysis	Venous, macroanalysis	Ascorbic acid added before laking	Macroanalysis after addition	Per cent recovery
T. H........	0.2		0.2			
G..........	0.5		0.5			
K..........	0.8		0.8			
W. H.......	0.2		0.3			
B..........		0.6	0.7	0.9	1.6	100
Z..........		0.6	0.8	0.9	1.7	100
C..........		1.2	1.3	0.9	2.2	100
P..........		1.0	1.1	1.0	2.3	110
S..........		1.2	1.3	0.8	2.1	100

TABLE III

Stability of Ascorbic Acid in Samples of Laked Whole Blood and Samples of Laked White Cells and Platelets

The results are expressed in mg. per 100 cc.

Subject	Immediate analysis	After standing 1 hr. at room temperature	After standing 24 hrs. in ice box
Laked whole blood			
F.	0.3		0.3
K.	0.4		0.4
"	1.4		1.4
V.	1.75*	1.75	
B.	1.60*	1.60	
T.	1.15	1.10	
Laked white cells and platelets			
P.	24.0		23.0
S.	36.0		34.0
M.	22.8		22.0

* 1 mg. of ascorbic acid per 100 cc. of blood added.

tion is decreased and above 4.7 the reaction is irregular and is not proportional to the concentration of ascorbic acid. Because the buffer capacity of the final solution containing the buffered dye and HPO_3 assures a

pH of between 4.3 and 4.5, error due to variation of pH is eliminated unless the reagents are not as specified.

Under the conditions specified in the procedures, we have found that methylene blue is not reduced by glucose, lactic acid, pyruvic acid, oxalic acid, uric acid, glutathione, cysteine, and sodium thiosulfate. Lund and Lieck (2) have found that creatine, creatinine, urea, adenine, guanine, hypoxanthine, xanthine, and ergothioneine do not interfere with the reaction.

The data of Table II show the agreement between macroanalyses of venous blood and microanalyses of venous and capillary blood. The data on recovery of ascorbic acid added to blood before laking with the glass-distilled water indicate that no loss of ascorbic acid occurs with the laking.

The data of Table III show that the solutions of such laked cells render the ascorbic acid relatively stable.

Attention has been called to the limitation of the concentration of plasma ascorbic acid as an index of the vitamin C nutrition of individuals whose plasma ascorbic acid is low (12). The determination of the ascorbic acid content of 0.2 cc. of capillary blood provides a convenient means of defining further the vitamin C nutritional state, as the concentration of whole blood includes the ascorbic acid content of the platelets and white blood cells.

SUMMARY

Methods are described for the analysis of the ascorbic acid content of whole blood, plasma, and the layer of white cell platelets of centrifuged oxalated blood by means of a photosensitized reaction with methylene blue. They are both more accurate and specific for the determination of ascorbic acid on small samples of blood than are the commonly used indophenol procedures and thus permit of more accurate microprocedures. Satisfactory analyses can be obtained with 0.2 cc. of capillary whole blood.

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A PHOTOELECTRIC METHOD FOR THE DETERMINATION OF PEPTIC ACTIVITY IN GASTRIC JUICE

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The need for a rapid, accurate method for the determination of peptic activity of gastric juice from clinical cases prompted the development of the method here reported. Procedures dependent upon chemical analysis, such as that of Anson and Mirsky,[1] are too time-consuming, while the widely used clinical method in which Mett's tubes are employed requires incubation for 24 hours and as a rule is not sufficiently accurate.

In principle, the method depends upon the use of a substrate consisting of a standardized, homogenized suspension of coagulated egg white. When acted upon by pepsin, the turbidity decreases with time. It is assumed that the amount of protein digested in unit time is proportional to this decrease in turbidity, and since turbidity can be measured by means of a photoelectric colorimeter, the peptic activity of the system can thus easily be determined. Since peptic hydrolysis of protein follows a monomolecular course, and since within limits its velocity constant is a function of the concentration of pepsin, it is convenient to express peptic activity as a velocity constant.

EXPERIMENTAL

Substrate—A fresh hen's egg is boiled for 10 minutes and the coagulated white is washed free of membrane and yolk and mashed in a mortar. With 5 ml. of water added per gm., the mash is passed through a homogenizer[2] several times. (With instruments of the Elvehjem type 2 drops of caprylic alcohol per 100 ml. may be needed to prevent foaming.) The volume is doubled with distilled water, homogenization is repeated, and the product is centrifuged at 1500 to 1800 R.P.M. for 15 minutes. After the removal of any surface film the supernatant homogenate is analyzed for total nitrogen and diluted to contain 0.5 mg. per ml. (which will usually mean approximately doubling the volume). Merthiolate is added to give a concentration of 1:10,000. Substrates thus prepared and stored below 10° have given reproducible results for 2 months.

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[1] Anson, M. L., and Mirsky, A. E., *J. Gen. Physiol.*, **16**, 59 (1933).

[2] An inexpensive, satisfactory instrument is carried by the Fisher Scientific Company, Pittsburgh, under the name of "laboratory homogenizer."

Standard Turbidity Curve—The relation of turbidimetric readings to protein concentration in the standard substrate is obtained as follows: With Filter 42 (blue-violet) for the Klett-Summerson instrument, a reading is taken of a 1:1 dilution of substrate in distilled water. With this as 100 per cent, the readings of eight to ten increasing dilutions are plotted against their respective percentage concentrations and a smooth curve is drawn through the points thus obtained. On the logarithmic scale of the Klett-Summerson apparatus the curve is nearly rectilinear, as shown in Fig. 1. *If the same instrument is used, subsequent batches of substrate may be adjusted to give the same 100 per cent reading as the first, thus eliminating*
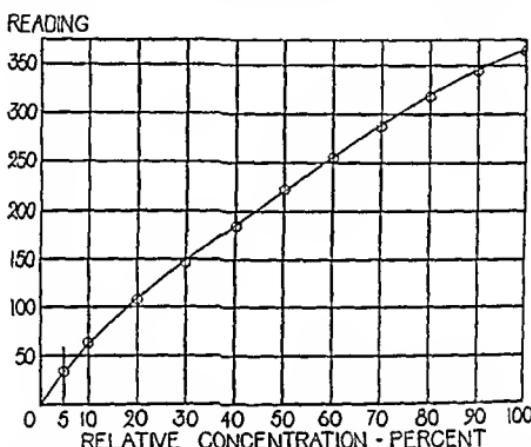
Fig. 1. Reading-concentration curve for a Klett-Summerson photoelectric colorimeter with a No. 42 filter. 100 per cent represents the concentration of substrate used in the digestion mixture.

further nitrogen determinations. The original curve will also be found to apply.

Buffer Solution—This consists of 0.2 N hydrochloric acid and 0.2 N (or 0.067 M) sodium citrate in the proportion 4:1. When diluted with substrate and distilled water to the concentration used in the digestion mixture, the pH is approximately 1.6. Sodium phosphate and hydrochloric acid in suitable proportions can as well be used.

Gastric Juice—The specimens are centrifuged at about 1500 R.P.M. for a minimum of 5 minutes and the relatively homogeneous middle layer is used for analysis. Because of the appearance of particles of food following the use of most test meals and variations in gastric emptying time, it is suggested that fractional drainage following insulin or histamine stimulation be employed. One of the authors (B. C. R.) has obtained satisfactory

results using 1 unit of insulin[3] per 10 pounds of body weight. Specimens are collected every 10 minutes for 50 minutes. The insulin is given intravenously and the test is concluded with the introduction through the stomach tube of about 100 ml. of a 50 per cent solution of Karo syrup, which has been found to counteract successfully any symptoms of hypoglycemia.

Specimens of gastric juice have been found to retain their activity without appreciable change for 12 hours at room temperature or for 48 hours if refrigerated immediately.

Procedure

A constant temperature water bath is set at $30° \pm 0.1°$. For preparation of the blank 1 ml. of the specimen is diluted with 4 ml. of the buffer and 5 ml. of distilled water and read in the colorimeter. Since the turbidity of gastric samples is often a function of pH, the buffer is added to reproduce the pH of the digestion mixture.

Two acid-cleaned tubes are set up, a colorimeter Tube A and a test-tube of similar size, Tube B. In Tube A are placed 5 ml. of the substrate and in Tube B are placed exactly 1 ml. of the centrifuged specimen and 4 ml. of the buffer. Both tubes are set in the water bath. When temperature equilibrium is reached (a minimum of 5 minutes), the contents of Tube B are poured into Tube A, the mixture into Tube B, and back to Tube A which is replaced in the water bath immediately. The time is taken at the beginning of the mixing, which should occupy 8 to 10 seconds. At 30 seconds the tube is placed in the colorimeter, read at exactly 1 minute, and replaced in the bath. It is again removed at $5\frac{1}{2}$ minutes and read at exactly 6 minutes. If in the case of a very weak specimen the concentration of undigested substrate is reduced by less than 10 per cent, the tube is replaced in the bath and the final reading is taken at some multiple of 5 minutes after the first, such as 30 minutes. Time is measured to within 2 seconds, the outsides of the tubes being dried carefully before mixing and before each reading.

Calculations

Since under the conditions of the assay protein hydrolysis follows a monomolecular course, the equation expressing the velocity of the hydrolysis is $dC/dt = kC$. C is the relative concentration of protein at the time t and k is the velocity constant, indicating the proportion of protein hydrolyzed per minute. On integration between two time limits t_1 and t_2 the velocity equation becomes $k = 1/(t_2 - t_1) \times 2.3 \log C_1/C_2$.

[3] Generously supplied by Eli Lilly and Company.

The two readings, after subtraction of the blank, are converted from the concentration curve to percentages of undigested substrate. If C_1 and C_6 are the values of these percentages at 1 and 6 minutes respectively, then the velocity constant is by substitution $k = 2.3/5 \log C_1/C_6 = 0.46 \log C_1/C_6$.[4] For the longer time intervals used with weak specimens, the result is divided by the multiple of 5 minutes between readings.

DISCUSSION

That the monomolecular velocity equation is followed under the conditions of the assay is shown in Fig. 2. The concentration of protein in the
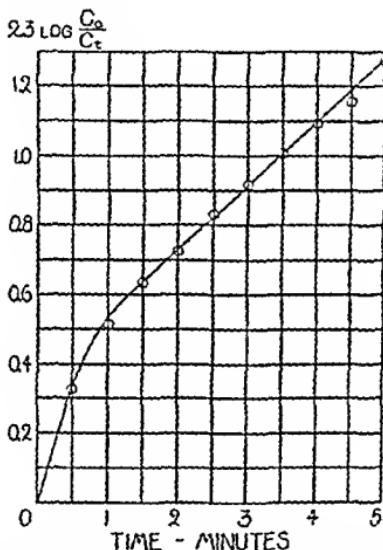
FIG. 2. Hydrolysis of a standard suspension of coagulated egg white by 0.2 per cent of Merck's granular pepsin at pH 1.6 and 30°. For the rectilinear part of the curve, $k = 1/t \, 2.3 \log C_0/C_t = 0.152$.

standard substrate in the presence of 0.2 per cent of Merck's granular pepsin was determined every 30 seconds. The plot of the values of $2.3 \log C_0/C_t$ against time is rectilinear after the 1st minute. The slope of the line is k. The reasons for the non-linearity of the 1st minute of the curve are not known. It is possible that the substrate is not completely homogeneous and the rate of hydrolysis of the smallest particles may be governed by factors not operating in the remainder of the substrate. The mechanical process of mixing is also a probable factor, since the length of this initially rapid period is not materially altered by extreme dilution of the enzyme, and is varied widely by variations in the time and method of mixing. In

[4] A nomogram converting the values of the two readings directly into k values has been found convenient and may be obtained upon request from the authors.

practice, therefore, the initial concentration is measured at 1 minute.   Furthermore, as might be anticipated, the curve falls off when observations are carried beyond 70 per cent of complete digestion.   The determinations are therefore reliable only if completed below this limit, which has been the case with all human gastric samples thus far encountered.   Fig. 2 also shows that within the limits defined above two readings will give sufficient accuracy for most purposes.

With a constant amount of enzyme the velocity constant of the reaction was found to increase rapidly with increasing dilution of the substrate. This was to be expected, since the substrate became relatively more saturated with the enzyme.   The concentration specified in the method was selected independently of this fact in order to utilize the most accurate range of the colorimeter.   Within this range, however, closely reproducible results

Table I
Degree of Reproducibility Possible in Determinations on Same Solution of Crystalline Pepsin

The solution of crystalline pepsin contained approximately 0.08 mg. per ml. of pepsin nitrogen. The same batch of substrate was used in all.

Determination No.	Velocity constant k	Per cent deviation from mean
1	0.0842	1.32
2	0.0824	0.84
3	0.0828	0.36
4	0.0824	0.84
5	0.0838	0.72
Mean	0.0831	0

have been obtained with variations up to 5 per cent in the substrate concentration.

The effect of inhibiting substances may be minimized by working with dilute solutions of the enzyme—indeed consistent determinations were made in the range of 1 to 10 γ of one commercial preparation of crystalline pepsin[5] with a 24 hour observation period. However, the desirability of a rapid method led to the selection of a concentration of gastric juice such that the determination can usually be completed in 6 minutes.   The variability under these circumstances remains within 3 per cent.   The reproducibility with crystalline pepsin is illustrated by Table I.

It was found that a variation of about 0.3° produced a maximum variation of about 4 per cent in the velocity constants at approximately 30°.   This

temperature was selected because it was found to lie in the range most conveniently controlled during the manipulations.

Variations in k due to pH changes from 1.4 to 1.8 were found to be less than 5 per cent. However, a buffer is used, because, since the protein substrate is insoluble, it does not itself function as a buffer.

The concentration of an enzyme is usually expressed in terms of some arbitrary unit, for it is rarely possible to determine the concentration in terms of weight. Moreover, the conditions of the assay must also be definitely specified. We have selected the velocity constant of the monomolecular hydrolysis of a standard protein homogenate determined under strictly
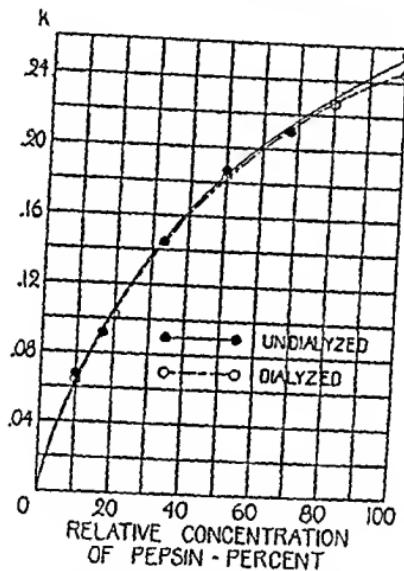
Fig. 3. Velocity constants of peptic hydrolysis with successive dilutions of Lehn and Fink crystalline pepsin, dialyzed until sulfate-free and undialyzed. In each case, 100 per cent is equivalent to approximately 0.5 mg. of pepsin nitrogen.

defined conditions as a convenient unit of peptic activity because it has certain advantages: (1) The velocity constant appears in the equation expressing the measured hydrolysis and hence requires no further definition. (2) It has physicochemical meaning, since it gives the proportion of the protein being hydrolyzed at any instant. (3) It is a function of the concentration of the enzyme. This is demonstrated in Fig. 3, where k is plotted against increasing concentrations of pepsin. We found, however, that k is not a *rectilinear* function of the concentration of crystalline pepsin. The almost exact coincidence of the two curves in this figure, although the dialyzable impurities have been removed in one preparation, seems to eliminate these impurities as an important factor in this connection. Our

results differ from those of Anson and Mirsky,[1] who by their method found a rectilinear relation between pepsin concentration and peptic activity. This non-linearity, however, is no real handicap to the use of the method for clinical purposes, since k still serves even at high levels as a sufficiently accurate measure of peptic activity.

TABLE II

Gastric Analysis 30 Minutes after Intravenous Insulin Stimulation

The ten patients were selected at random from the hospital wards, with no evidence of digestive disease. Acidity is measured by the glass electrode method.

Patient No.	Sex	Age	Color	Diagnosis	Gastric analysis	
					pH	k
		yrs.				
1	M.	20	White	Orchiopexy	2.77	0.202
2	"	41	Negro	Inguinal hernia	2.30	0.114
3	"	35	"	" "	7.14	0.000
4	"	35	White	Knee injury	2.01	0.251
5	"	46	"	Hemorrhoids	6.08	0.130
6	"	56	"	Neurasthenia	1.89	0.006
7	F.	52	Negro	Carcinoma of vulva	7.14	0.288
8	"	37	"	Myoma uteri, preoperative	1.55	0.325
9	"	57	"	" " postoperative	2.09	0.212
10	"	39	White	Finger infection	2.13	0.225

TABLE III

Fractional Gastric Analysis after Intravenous Insulin Stimulation

The subject was a 43 year-old white male with a healed gastric ulcer, receiving no medication. The flow of juice ceased after 30 minutes.

Specimen time	Volume	pH	Pepsin k
min. after insulin	*ml.*		
Fasting	13	7.40	0.005
10	5	6.49	0.048
20	13	5.96	0.027
30	3		0.015
40	0		

However, determinations made on the highly active juice from Pavlov pouches[6] showed so rapid a decrease in turbidity that it was necessary to use 1 ml. of a 1:4 dilution of the specimen. Under these circumstances the k values varied from 0.15 to 0.30. Extrapolation of the curve in Fig. 3

[6] Kindly supplied by Dr. M. H. F. Friedman, Jefferson Medical College, Philadelphia.

shows that these results cannot simply be multiplied by the factor of the dilution to be comparable with the k values obtained from human specimens. This is a distinct limitation of the method, although if the same dilution is used in all specimens from a given source the results will be comparable for that source.

No attempt has been made to relate k to units of measurement obtained by other methods. If such a relationship is desired, it could easily be obtained by comparison of an activity-concentration curve such as that of Fig. 3 with a similar activity-concentration curve obtained by any other method.

In the presence of regurgitated bile sufficient to give marked coloration, the substrate has occasionally been observed to flocculate during the digestion, making it necessary to repeat, or even discard the determination. The other most frequently met sources of error, in the order of their apparent importance, are variations in the time and method of the manipulations— particularly the mixing, cleanliness of the glassware, and errors in reading the colorimeter due to imperfect timing.

Tables II and III show the results of single analyses 30 minutes after insulin stimulation and one fractional analysis after insulin stimulation. These values are given merely as an orientation of the values that may be expected, and do not imply a level of normality. Further study on the clinical application of the method is being made by one of the authors (B. C. R.).

SUMMARY

A method for the estimation of peptic activity is described which combines rapidity with accuracy, and is particularly adapted to clinical studies.

Enzyme activity is measured photoelectrically as the decrease in turbidity of a standardized, homogenized suspension of coagulated egg white under specified conditions. Accurate measurement is possible down to a level equivalent to $1\,\gamma$ of crystalline pepsin.

Protein hydrolysis so measured follows a monomolecular course and hence the enzyme activity is expressed as a velocity constant.

The velocity constant is a curvilinear function of pepsin concentration and serves throughout the range encountered clinically as a convenient measure of the peptic activity of gastric samples.

Representative clinical results are given.

THE SYNTHESIS AND PROPERTIES OF NINHYDRIN UREIDE

By DONALD D. VAN SLYKE AND PAUL B. HAMILTON

(From the Hospital of The Rockefeller Institute for Medical Research, New York)

Evidence that ninhydrin (triketohydrindene hydrate) combines with urea was first noted by Van Slyke, Dillon, MacFadyen, and Hamilton (1) who observed that CO_2 evolution by spontaneous hydrolysis of urea at 100° in buffered water solutions at pH 2.5 and 4.7 was retarded by the presence of ninhydrin. It was concluded that combination of ninhydrin with urea must occur to form a compound which at 100° evolves CO_2 more slowly than does urea. In the present work the formation of a stable ninhydrin-urea compound has been confirmed by its preparation in crystalline form. The conditions for obtaining maximal combination of urea with ninhydrin have been studied.

The ureide obtained is a colorless crystalline substance, $C_{10}H_{10}O_5N_2$, formed by combination of 1 molecule of ninhydrin with 1 of urea. It is somewhat soluble in hot alcohol or glacial acetic acid, insoluble in chloroform. It can be recrystallized from 10 parts of hot water. The product readily loses 7.6 per cent of water *in vacuo* at 56°, yielding $C_{10}H_8O_4N_2$, which melts with decomposition at 216–217° (uncorrected). Whether the loss is of water of crystallization or is due to anhydride formation has not been determined.

At room temperature in water solution the ureide does not show measurable quantitative reactions for urea, such as hydrolysis with urease (2) or decomposition with hypobromite (3). When heated in water solution, the ureide slowly undergoes partial degradation or hydrolysis with evolution of CO_2. Free urea can be demonstrated with urease, and the CO_2 may originate from heat hydrolysis of this urea. Some ninhydrin also appears to be set free at 100°. The possibility of other degradation products has not been excluded.

The ability of ninhydrin to combine with urea is of practical utility when it is desired to remove urea in order to prevent its interference with the determination of other substances. The use of ninhydrin in this manner preliminary to determination of the amino acids in blood filtrates has been described by the writers (4).

<div align="center">EXPERIMENTAL</div>

Synthesis of Ninhydrin Ureide—16 mM, 2.84 gm., of ninhydrin and 32 mM, 1.920 gm., of urea were dissolved in 80 cc. of 0.1 N sulfuric acid and

gently boiled under a reflux for 15 minutes. On cooling in the ice box, 2.798 gm. of crystalline product separated. The product was redissolved with warming and allowed to crystallize slowly from 10 parts of water, and the crystals were collected on a Buchner funnel, washed with cold water, and dried *in vacuo* at room temperature over calcium chloride. The finely pulverized crystals contained 7.6 per cent water, which was removed by drying *in vacuo* at 56° for 30 minutes. No further loss occurred *in vacuo* at 110° in 4 hours.

When the well pulverized crystals were heated in a melting point tube at the rate of 1° rise in 3 seconds, the slight darkening observed to begin at 180° became very marked at 214°, and the substance decomposed sharply with effervescence at 216–217° (uncorrected).

$$C_{10}H_8O_4N_2.\quad \text{Calculated.}\quad C\ 54.6,\ H\ 3.64,\ N\ 12.76$$
$$\text{Found.}\quad\ \ "\ 54.45,\ "\ 3.83,\ "\ 12.60$$

Properties of Ninhydrin Ureide—At room temperature in aqueous 0.01 N sulfuric acid (pH 2) the ureide is stable, giving no reaction with alkaline hypobromite or with urease.

Aliquot portions of a 0.94 per cent solution of the dried crystalline ureide ($C_{10}H_8O_4N_2$) were dissolved in 0.01 N sulfuric acid with warming to 60°. The solution was cooled to room temperature and 5 cc. aliquots treated with alkaline hypobromite as for quantitative urea analysis (3). No measurable amounts of nitrogen were evolved. Similarly 5 cc. aliquots of the same solution were analyzed for free urea with urease as in quantitative urea analysis by this procedure (2). No measurable amounts of CO_2 were evolved.

When the ureide is heated at 100° in aqueous solution at pH 2, slow evolution of CO_2 takes place. In Fig. 1 moles of CO_2 evolved per mole of ureide from a 0.94 per cent solution of the latter (42.6 mM) at pH 2 are plotted as ordinates against time of heating as abscissae. Contrary to most reactions, the rate of CO_2 evolution increased with time. The lag may be explainable by the requirement of more than an hour for dissociation of the ureide at 100° to approach its maximum. The observation most pertinent to the present study is that the ureide evolves very little CO_2 during the first 30 minutes of heating, being only about 1 per cent of the CO_2 evolved from an equivalent amount of free urea under the same conditions.

Some evidence that urea and ninhydrin are also products of its degradation at 100° was obtained by treating solutions of the ureide heated at 100° for 60 minutes at pH 2 with urease, alkaline hypobromite, and excess amino acid.

Aliquot portions of a 0.94 per cent solution of the ureide were heated in 0.01 N sulfuric acid (pH approximately 2) at 100° for 60 minutes, then cooled quickly to 20°,

and 5 cc. aliquot portions were treated with alkaline hypobromite (3) and with urease (2). Nitrogen was evolved to an extent of 2.1 per cent of the nitrogen of the ureide, and with urease CO_2 was evolved to an extent of 5.6 per cent of the urea in the ureide. To other aliquot portions of the same solution a large excess of pure α-alanine was added and the solution heated at 100° for a period of 20 minutes. Evolved CO_2 was measured as in the ninhydrin-CO_2 method (1) and found to be equivalent to about 30 per cent of that evolved by free ninhydrin under similar conditions.

The evidence here presented would seem to indicate that the ureide does in fact dissociate into its initial components and that the reaction is therefore a reversible one. The data do not, however, suffice to prove this possibility, owing partly to lack of precise quantitative relationships and partly to the specificity of some of the methods employed. No attempt has been made to isolate the products of degradation or hydrolysis.
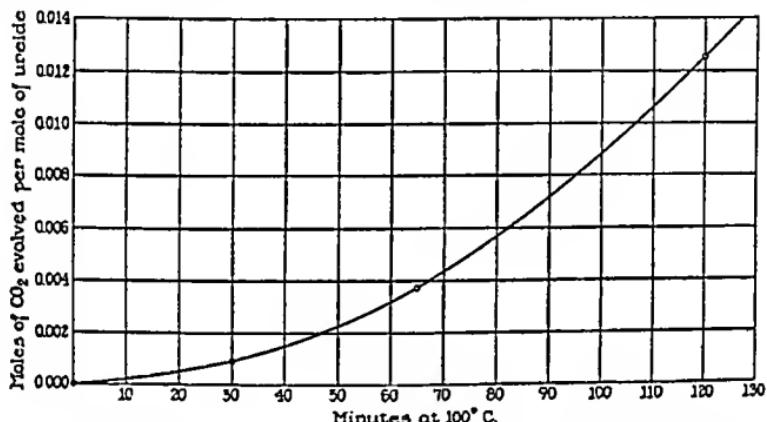
Fig. 1. Rate of evolution of CO_2 from ninhydrin ureide heated at 100°

Retarding Effect of Ninhydrin on Evolution of CO_2 from Urea at 100°

Urea, 1.285 mg. per cc. (21.4 mM), was dissolved in an aqueous solution of 0.5 per cent picric acid adjusted to pH 2.0 (glass electrode) with NaOH, simulating in picric acid content and pH the blood and plasma filtrates used by Hamilton and Van Slyke (4) for amino acid determinations. Portions of 5 cc. of the solution were pipetted into all-glass reactions vessels ((4) Fig. 1, A) and varying weighed amounts of ninhydrin were added to give the concentrations, 10, 20, and 40 mg. per cc. (56, 112, and 224 mM), indicated on the curves of Fig. 2. The vessels were evacuated as described for filtrate analyses (4) and were immersed in a vigorously boiling water bath (100°) for the varying lengths of time indicated. They were then cooled in cold water and the CO_2 formed was determined as described by these authors (4).

From the shape of the curves of Fig. 2 it can be seen that the effect of ninhydrin in retarding CO_2 formation from urea is not great for the 1st minute but increases progressively till at the end of 20 minutes the rate of CO_2 evolution falls to a rate which varies inversely approximately as the

square of the ninhydrin concentration. This rate in the presence of 1, 2, and 4 per cent concentrations of ninhydrin was 0.17, 0.05, and 0.01, respectively, as great as in the absence of ninhydrin. That ninhydrin takes some time to exert its full effect in depressing the rate of CO_2 formation is attributable to the fact that this interval of time is necessary for combination of ninhydrin and urea to approach its maximum.

Even after 20 minutes, slow evolution of CO_2 continues and is compatible with the supposition that formation of the ureide is a reversible
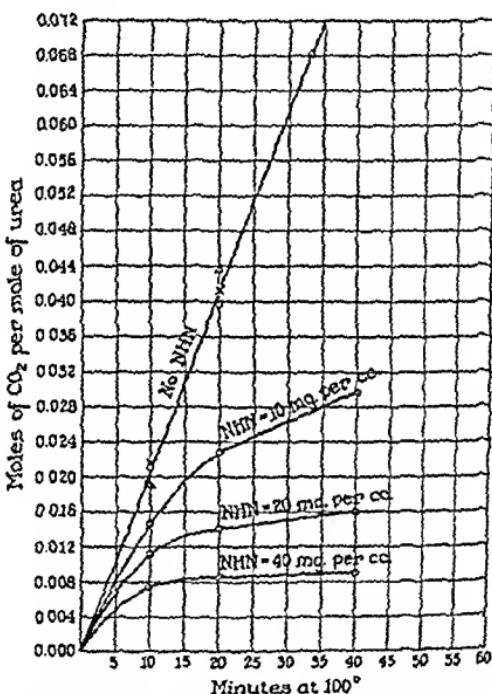
Fig. 2. Rate of evolution of CO_2 from urea heated at 100° in 0.5 per cent picric acid solution, pH 2, and in the presence of concentrations of ninhydrin varying from 0 to 40 mg. per cc. The symbols O, $\times$, $\triangle$ indicate results of separate experiments.

reaction, and that at 100° measurable dissociation of the urea from the ureide occurs unless a large excess of ninhydrin is present. When the ninhydrin concentration is 40 mg. per cc., evolution of CO_2 is depressed almost to zero, as indicated by the nearly horizontal bottom curve of Fig. 2.

Velocity of Combination of Ninhydrin and Urea at Different Temperatures

The velocity of combination of ninhydrin and urea was investigated at 26°, 47°, 61°, and 75° to determine the most practicable conditions of time and temperature for the elimination of free urea from solution.

At each temperature a series of test-tubes was charged with 5 cc. each of 0.01 N H_2SO_4, followed by weighed samples of urea and ninhydrin to give concentrations of 9.90 and 222 mM (3.96 per cent) respectively. The mixtures were incubated at the above temperatures with continuous rocking. After 1, 2, 3, and 4 hour intervals the tubes were removed from the rocker and their contents transferred to the Van Slyke-Neill manometric gas chamber where the uncombined urea was determined with alkaline hypobromite (3).

In Fig. 3, the per cent of urea remaining uncombined with ninhydrin is plotted as ordinates against the time of incubation as abscissae for the temperatures indicated on each of the family of curves.
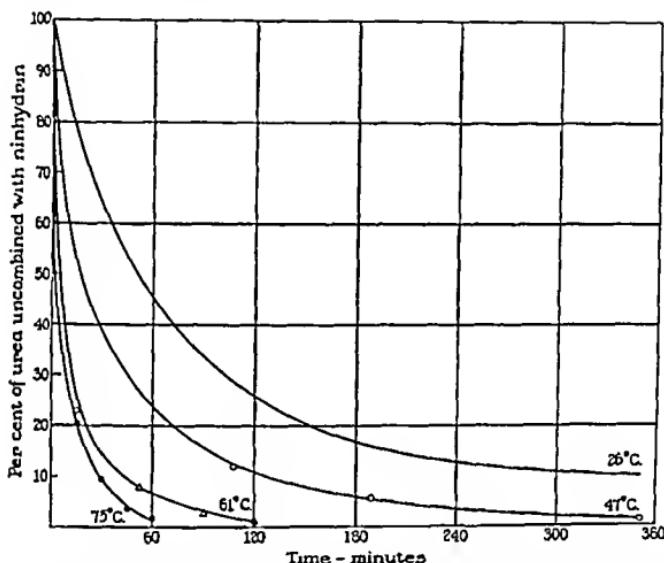
FIG. 3. Per cent of urea uncombined with ninhydrin plotted as ordinates against time of incubation in minutes at 26°, 47°, 61°, and 75° as abscissae.

The data obtained in these experiments form the basis upon which Hamilton and Van Slyke (4) have fixed conditions to prevent interference by urea when amino acids in blood filtrates are determined by the ninhydrin-CO_2 method.

Possibility of Condensation of Ninhydrin with Amino Groups of Amino Acids

The effect of ninhydrin in making completely labile the CO_2 of the carboxyl groups of α-amino acids, but not of other organic acids, is the basis of the specificity of the ninhydrin-CO_2 analytical method for free α-amino acids (1). Ninhydrin transforms the amino acid to some intermediary product in which the carboxyl group is labile. In view of the instability of α-keto acids, one might have assumed the labile intermediary to be a keto

acid, formed by oxidizing the $CH(NH_2)$ group to CO; this assumption would have been consistent with the fact that the end-product from $R \cdot CH(NH_2) \cdot COOH$ is the aldehyde, $R \cdot CHO$. Nevertheless it appears that the α-keto acid is not an intermediary, because pyruvic acid has been found by us to evolve its carboxyl CO_2 only about 0.1 as rapidly as alanine under the influence of ninhydrin. The combination of 1 mole of ninhydrin with 1 mole of urea suggests that ninhydrin reacts similarly with the NH_2 groups of the α-amino acids, and that the combination may labilize the carboxyl group. That condensation occurs with the NH group of proline and hydroxyproline has been shown by Grassmann and von Arnim (5). We have tried to isolate the intermediary hypothetical condensation product of alanine, but the solubility and instability of the product have thus far prevented its isolation.

SUMMARY

Ninhydrin and urea combine to form a stable crystalline ureide of the composition $C_{10}H_{10}O_5N_2$, 1 mole of urea combining with 1 mole of ninhydrin without loss of water. The compound readily loses 1 mole of water at $56°$ to form a substance of the composition $C_{10}H_8O_4N_2$.

From the velocity of the combination conditions have been defined which enable one to remove urea from solution nearly quantitatively by formation of the ureide.

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THE ANALYSIS OF EIGHT AMINO ACIDS BY A MICROBIOLOGICAL METHOD*

Sirs:

The authors' microbiological method is based on the production of lactic acid by *Lactobacillus arabinosus* 17-5 in a synthetic medium composed of amino acids, dextrose, adenine, guanine, uracil, sodium acetate, mineral salts, and vitamins. The composition of the authors' medium, which was a modification of that employed by Pollack and Lindner,[1] and the amino acid requirements of *Lactobacillus arabinosus* have recently been reported by one of us.[2]

Experimental Data from Microbiological Assay of Eight Amino Acids

Amino acid*	Grade	Limiting amounts	Amount present	Amount reported	Deviation between amounts present and reported
		mg.	mg.	mg.	per cent
l(−)-Cystine†	C.P.	40–100	44.5	42 ± 2	−4
dl-Isoleucine†	A.P.	40–100	100.1	95 ± 2	−5
dl-Methionine†	"	40–100	39.7	41 ± 1	+3
l(−)-Leucine†	"	100–175	150.2	150 ± 3	−0.2
dl-Valine†	"	100–175	125.3	124 ± 4	−1
l(+)-Glutamic acid†	"	200–400	351.5	358 ± 15	+2
dl-Threonine‡	C.P.	200–400	199.8	187 ± 20	−6
l(−)-Tryptophane†	"	40–100	68.6	71 ± 1	+4
dl-Lysine monohydrochloride†	A.P.		101.3	§	
l(−)-Tyrosine†	"		49.8	§	
dl-Phenylalanine†	"		79.5	§	

* The optical forms listed were used as standards and unknowns.

† Obtained from Amino Acid Manufactures.

‡ Obtained from Merck and Company, Inc.

§ Results inconsistent.

Quantities of eleven C.P. or analytically pure grade amino acids ranging from approximately 50 to 350 mg. were weighed accurately and mixed.

[1] Pollack, M. A., and Lindner, M., *J. Biol. Chem.*, **143**, 655 (1942).

[2] Shankman, S., *J. Biol. Chem.*, **150**, 305 (1943).

The mixture was assayed for each of eight amino acids by means of standard microbiological techniques. In order to save materials and time, which would have been required to ascertain levels of amino acids, the assayist was informed of the limiting weights of each amino acid in the mixture. The quantity of each amino acid present in the mixture was estimated from the titration data and standard curves for lactic acid production at varying levels of each of the eight amino acids. The experimental data and the results of the analysis are shown in the table.

It may be observed that the average deviation of the quantities of amino acids found differed from that of the amino acids present by 3.2 per cent. It was concluded that the accuracy of this analysis compares favorably with that of other microbiological assays. It is evident, also, that the authors' assay procedure is rapid, convenient, and dependable. It seems probable that comparable procedures for the assay of other amino acids can be found and that there are many types of investigations in which amino acids can be analyzed satisfactorily by microbiological methods.

Wm. T. Thompson Company   S. Shankman
  Los Angeles   Max S. Dunn
Chemistry Department   Louis B. Rubin
  University of California
  Los Angeles

OBSERVATIONS WITH P³² OF THE CHANGES IN THE ACID-SOLUBLE PHOSPHATES IN THE LIVER COINCIDENT TO ALTERATIONS IN CARBOHYDRATE METABOLISM

Sirs:

Recent studies in this laboratory with radioactively labeled phosphorus on the acid-soluble phosphates of liver have yielded interesting information regarding the phosphate cycle in the intact animal. We wish to report findings which indicate the marked influence of insulin and of glucose administration on the phosphate compounds of liver.

Distribution of Labeled P in Acid-Soluble Phosphates of Liver

Treatment	No. of rats	Total acid-soluble	Inorganic	P of adenosine triphosphate		Mercury* ppt. P	Alco-holt ppt. P	Residu-al‡ P
				Labile	Non-labile			
Controls fasted 12 hrs.	10	177	77.6	11.7	3.3	20.2	44.0	28.6
		±12.6	±8.3	±2.9	±2.4	±5.1	±5.1	±4.5
Glucose (300–400 mg. intraperitoneally)	10	204.0	56.6	49.0	12.8	24.6	25.4	12.9
		±14.2	±7.5	±7.2	±3.3	±3.8	±3.1	±3.8
Insulin (4 units intraperitoneally)	8	244.3	92.5	41.5	15.6	21.4	26.2	14.5
		±19.2	±9.6	±8.1	±4.0	±5.6	±7.6	±3.1
Glucose + insulin (injected amounts same as above)	6	262.0	83.4	64.5	16.2	25.0	22.7	20.2
		±18.3	±8.0	±6.8	±2.8	±9.0	±6.7	±3.5

The analyses were carried out on rats sacrificed 110 minutes after administration of Na_2HPO_4 labeled with P³². All values are in per cent of administered dose × 10 of the labeled P per 100 gm. of fresh liver. The measure of variability is the mean deviation from the mean.

* This fraction contains nucleotides other than adenosine triphosphate.

† This fraction consists largely of glycerol phosphate but it also contains some hexose monophosphates.

‡ This fraction consists largely of phosphoglyceric acid.

Rats weighing about 200 gm. were fasted for a period of 12 hours, given the treatment shown in the table, and then injected intraperitoneally with trace doses of $Na_2HP^*O_4$.[1] The animals were sacrificed at varying time intervals and the acid-soluble phosphate constituents were fractionated and analyzed for radioactivity. The distribution of radioactive phosphorus in the various acid-soluble fractions of the liver 110 minutes after administration of the phosphate is summarized in the table.

A maximum in the P* concentration of the total acid-soluble phosphates

[1] The chemical symbol with an asterisk is used to represent an element labeled with radioactive isotope.

was usually attained at about 110 to 120 minutes. Glucose administration caused a marked alteration in the time relations of the labeled phosphate distribution. The peak in the radioactivity of the labile P of adenosine triphosphate of the animals given glucose occurred at 110 minutes after the injection of P*, whereas the peak in the control group was at 200 minutes. The peak in the radioactivity of the non-labile P of the adenosine triphosphate was similarly reduced from 250 to 110 minutes. The peak in the radioactivity of the P of the alcohol and the residual fractions was increased in time by glucose administration from 110 to 210 and 245 minutes respectively.

The data of the table show that both glucose and insulin produce an increase in the adenosine triphosphate P* and a decrease in the residual and alcohol P*. Insulin induces a marked rise in the total acid-soluble and inorganic P*. This confirms previous findings of Nelson and coworkers[2] that insulin causes a shift of phosphate from serum to liver. Insulin and glucose administered together produce a significantly greater increase in the radioactivity of the labile P of adenosine triphosphate than either does alone.

It was found that the injection of malonate, phlorhizin, or sodium fluoride prevented the rise in the labile P* of adenosine triphosphate following administration of glucose; iodoacetate was without effect. The dietary state of the animal was also found to have a profound influence on the distribution of the radioactive phosphate.

These results illustrate the importance of the rôle played by the organophosphates in the metabolism of the liver *in vivo*.

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[2] Nelson, N., Rapoport, S., Guest, G. M., and Mirsky, I. A., *J. Biol. Chem.*, **144**, 291 (1942).

GLUTAMINE AS SOURCE MATERIAL OF URINARY AMMONIA

Sirs:

Nash and Benedict[1] in 1921 showed that ammonia excreted in the urine is formed in the kidneys. The source material has been in dispute; at first it was believed to be urea, then amino acids.

Dogs have been prepared with kidneys explanted by the technique of Rhoads,[2] which renders it possible without anesthesia to draw blood from the renal vein by skin puncture. The renal blood flow was estimated from the creatinine excretion by the principle of Van Slyke, Rhoads, Hiller, and Alving.[3] The cc. of renal blood flow per minute were calculated as (mg. of creatinine excreted per minute)/(mg. removed from 1 cc. of renal blood). The amount removed from renal blood was calculated as the difference in creatinine content per cc. between arterial and renal venous bloods. From the renal blood flow thus obtained and the arterio-renal-venous differences of other substances, such as urea and α-amino nitrogen, the amounts of such substances removed from the blood by the kidneys per minute could be calculated.

All the urea thus found to be removed from the blood by the kidneys was excreted unchanged in the urine. Even when the ammonia excretion was made greater than the urea, by giving HCl and a low protein diet, none of the urea extracted from the blood by the kidneys was used to make ammonia. The same was true of adenosine and adenylic acid. Of α-amino N,[4] sometimes none was removed from the blood by the kidneys and sometimes small amounts, inadequate to provide nitrogen for the ammonia excreted.

The amide nitrogen of glutamine, demonstrated by one of the writers[5] in blood, was removed from the blood plasma in much greater amounts than appeared in the urine; the excess sufficed to provide (*a*) the ammonia removed from the kidney via the renal vein[1] and (*b*) 60 per cent or more of the ammonia excreted in the urine. Administration of glutamine to a dog in hydrochloric acid acidosis markedly increased the ammonia excretion. Depressing the ammonia excretion by changing from hydrochloric acid acidosis to bicarbonate alkalosis was accompanied by a corresponding decrease in removal of glutamine from the renal blood. The glutamine

[1] Nash, T. P., Jr., and Benedict, S. R., *J. Biol. Chem.*, **48**, 463 (1921).

[2] Rhoads, C. P., *Am. J. Physiol.*, **109**, 324 (1934).

[3] Van Slyke, D. D., Rhoads, C. P., Hiller, A., and Alving, A. S., *Am. J. Physiol.*, **109**, 336 (1934).

[4] Hamilton, P. B., and Van Slyke, D. D., *J. Biol. Chem.*, **150**, in press (1943).

[5] Hamilton, P., *J. Biol. Chem.*, **145**, 711 (1942).

analyses were duplicated by two methods: (1) measuring the decrease in α-amino N caused by heating at pH 6.5;[5] (2) measuring the NH_3 formed by action of a glutaminase prepared by one of the writers (R. M. A.) from kidney tissue. In some experiments in which the amount of glutamine amide nitrogen removed from the blood did not suffice to account for all of the urinary ammonia, the small amounts of α-amino N removed sufficed to make up the difference. Some results indicating the relation between glutamine and ammonia are given in the table.

Condition	Renal blood plasma flow	Urine ammonia N	Glutamine amide N removed from renal blood	Preformed NH₂-N in blood	
				Arterial	Renal venous
	cc. per min.	*mg. per min.*	*mg. per min.*	*mg. per 100 cc.*	*mg. per 100 cc.*
Acidosis	245	0.562	0.33	0.02	0.10
	268	0.605	0.39	0.02	0.10
	262	0.615	0.41	0.04	0.10
Alkalosis	178	0.005	0.02	0.034	0.075
	200	0.004	0.02	0.039	0.075
	191	0.004	0.04	0.042	0.063

It appears that the data reported provide the first direct evidence by experiments *in vivo* of a physiological function of glutamine in animals.

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A RAPID METHOD OF ESTIMATING N¹-METHYLNICOTIN-AMIDE IN URINE*

Sirs:

Having established that N¹-methylnicotinamide is the chief excretion product of nicotinic acid metabolism in human urine,[1,2] we have made a large number of determinations employing a modification of Najjar and Holt's method described elsewhere in this *Journal*.[1]   Although the method is quite simple, the time required for the laborious preparation and washing of the zeolite columns limits its usefulness.   A number of trials have shown that without any appreciable sacrifice in accuracy the adsorption on and elution from the zeolite columns may be omitted and the fluorescent substance determined directly in butanol extracts of the urine, literally in a few minutes.   This direct method is particularly applicable to saturation or tolerance tests after a dose of nicotinic acid or of its amide.

The urines in such tests are best diluted 10-fold with 25 per cent KCl solution.   To a 10 ml. aliquot of the diluted urine in a test-tube, made alkaline with 2 or 3 drops of 10 N NaOH solution, 0.5 gm. of Lloyd's reagent is added, the contents mixed for 1 minute by inversion, centrifuged for 2 to 3 minutes, and the supernatant fluid decanted.   This procedure was shown to destroy completely the fluorescence of N¹-methylnicotinamide, without affecting the other pigments of urine which are not adsorbed at the alkaline pH.   Hence this proved to give the best blank values we have been able to obtain so far.   An aliquot of 1 to 5 ml. of the diluted urine containing 3 to 15 γ and simultaneously an equal volume of the blank prepared as above are placed in 125 ml. separatory funnels, extracted with n-butanol, and the fluorescence measured exactly as described in our previous article.[1]

In the table are given values for N¹-methylnicotinamide in urines of six patients, collected for 14 hours after an oral dose of 500 mg. of nicotinamide. When compared, the figures obtained by fluorescence analyses directly and after adsorption on and elution from permutit show substantial agreement.

"Trigonelline" values are included for comparison; these were obtained by the older colorimetric method[3] and include both the betaine trigonelline and N¹-methylnicotinamide.   These figures and other data on hand indi-

[1] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **150**, 395 (1943).

[2] Sarett, H. P., *J. Biol. Chem.*, **150**, 159 (1943).

[3] Perlzweig, W. A., Levy, E. D., and Sarett, H. P., *J. Biol. Chem.*, **136**, 729 (1940).

cate that in human urine N^1-methylnicotinamide comprises practically all of the methylated nicotinic acid previously determined and designated as trigonelline. It should also be noted that since trigonelline does not affect the fluorescence measurement, it is not necessary to omit trigonelline-containing foods (coffee, legumes) when nicotinic acid excretion tests are conducted by the above method.

Subject No.	Direct method	Permutit method	"Trigonelline" method
	γ per ml.	γ per ml.	γ per ml.
1	41	41	47
2	39	40	39
3	40	48	47
4	113	127	117
5	106	109	106
6	45	45	41

The direct method is also applicable to urines of subjects not receiving nicotinic acid other than dietary. Thus in three normal subjects urines collected 2 hours after breakfast gave the following N^1-methylnicotinamide fluorescence values obtained by the direct and permutit methods, in micrograms per ml.: 6.0 and 5.75, 10.6 and 10.0, 6.0 and 5.9, respectively. In urines with a much lower concentration, 0 to 2γ per ml., the values become quite uncertain, regardless of the method employed, and may be useful only for comparative purposes. Such urines are analyzed directly without dilution.

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EFFECT OF GLUTAMIC ACID ON THE FORMATION OF ACETYLCHOLINE*

Sirs:

Recent investigations suggest that the release of acetylcholine (ACh) is intrinsically connected with the potential of the nerve action.[1] The release and breakdown of ACh are but the first step in a chain of reactions, the "acetylcholine cycle," as has been shown in experiments on the electric organ of *Electrophorus electricus*.[2] ACh is resynthesized by the free energy of phosphocreatine, adenosine triphosphate (ATP) acting as the intermediate link as in muscle. As a result of these findings, an enzyme, choline acetylase, was isolated from brain, which in the presence of ATP in cell-free solution and *under anaerobic* conditions synthesizes ACh.[3]

On dialysis, the activity of choline acetylase decreases rapidly (Nachmansohn and Machado). Whereas in the original extracts 40 to 80 γ of ACh are synthesized per gm. in 90 minutes, only 4 to 6 γ of ACh are formed after a dialysis of 2 hours.

Clinical observations suggest that glutamic acid has a favorable effect on epileptic patients suffering from petit mal attacks, whereas patients with grand mal appear to be not affected.[4] On the hypothesis that the slow waves which appear in the electroencephalogram during attacks of *petit mal* may in some way be connected with a lowered rate of ACh formation, the effect of glutamic acid has been tested on ACh synthesis in dialyzed extracts of rat brain. (For the experimental procedure, see Nachmansohn and Machado.[3])

Addition of the naturally occurring $l(+)$-glutamic acid to dialyzed extracts in 2×10^{-2} M concentration increases the rate of formation of ACh by about 4 to 5 times. At 1×10^{-2} M concentration, the increase is about 3 to 4 times. $d(-)$-Glutamic acid has a small effect. The effect of glutamic acid is stronger than that of other amino acids tested so far. $l(+)$-Aspartic acid and dl-serine have no effect. dl-Alanine and dl-methionine and glutamine increase the rate of formation of ACh about twice at a concentration of 2×10^{-2} M. Of some other dicarboxylic acids tested, no effect was found

[1] Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, **5**, 499 (1942). Fulton, J. F., and Nachmansohn, D., *Science*, **97**, 569 (1943).
[2] Nachmansohn, D., Cox, R. T., Coates, C. W., and Machado, A. L., *J. Neurophysiol.*, in press.
[3] Nachmansohn, D., and Machado, A. L., *J. Neurophysiol.*, in press.
[4] Price, J. C., Waelsch, H., and Putnam, T. J., *J. Am. Med. Assn.*, **122**, 1153 (1943).

with *l*-malic, malonic, and α-ketoglutaric acids, whereas succinic acid increases the rate about twice. In view of the action of succinic acid, citric acid has been tested. This tricarboxylic acid increases the rate of ACh formation in dialyzed extracts about 4 to 6 times.

The question arises whether glutamic acid or citric acid or similar compounds are coenzymes of choline acetylase forming part of that enzyme system. The action of these naturally occurring compounds on ACh formation appears of interest, especially in view of the clinical effect observed with one of them in disorders of nervous function.

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INDEX TO AUTHORS

INDEX TO SUBJECTS